# Fatty acid bioconversion capacity of rainbow trout (*Oncorhynchus mykiss*): temperature and nutritional modulation

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# Summary

Since several decades, a specific attention has been drawn on the production of fish with a high content in health-promoting omega-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA) in aquaculture. This challenge has to be met considering the replacement of the finite and expensive fish oil used in fish feeds by alternative lipid sources. Plant-derived oils are considered as promising alternatives but, since they lack n-3 LC-PUFA, the n-3 LC-PUFA content of fish is reduced. Nevertheless, some fish species, especially salmonids, possess a relatively high capacity to endogenously biosynthesise n-3 LC-PUFA from the substrate α-linolenic acid (ALA) when ALA-rich plant-derived oils are included to their feed. Different feeding strategies are therefore implemented in order to stimulate the biosynthesis capacity of fish and consequently increase their n-3 LC-PUFA content. However, it is worth noting that the increased temperature of the supply water, induced by the global warming, may interfere with this bioconversion capacity. To date, little is known regarding the impacts of increased water temperature on the fish fatty acid bioconversion capacity and research is still required in the development of efficient and sustainable feeding strategies.

The present study aimed at investigating the impacts of an increased water temperature and two feeding strategies on the fatty acid bioconversion capacity of the salmonid species rainbow trout (*Oncorhynchus mykiss*) through *in vivo* feeding trials and *in vitro* approaches. In a first experimental part, fish were subjected to a 4°C-increase water temperature and fed a control diet based on fish oil or an experimental diet based on ALA-rich linseed oil. The increased water temperature induced negative effects on the fatty acid bioconversion capacity of trout fed a linseed oil diet, both on the gene expression of fatty acid desaturase 2 (*fads2*) and the activity of Δ6 fatty acid desaturase involved in the n-3 LC-PUFA biosynthesis. Interestingly, only a slight negative effect of temperature was observed on the fish n-3 LC-PUFA content. In contrast with the temperature effect, the replacement of fish oil by linseed oil induced an increased fatty acid bioconversion capacity but significantly decreased the fish n-3 LC-PUFA content. In a second experimental part, we evaluated the impact of a pre-experimental omega-3 polyunsaturated fatty acid (n-3 PUFA) depletion of fish on the fatty acid bioconversion capacity of rainbow trout fry when subsequently fed an ALA-

rich linseed oil diet. This study demonstrated that the basal high capacity of rainbow trout to biosynthesise n-3 LC-PUFA was not modified after a fish n-3 PUFA depletion. In a third experimental part, the sesame seed and linseed lignans and the enterolignan enterodiol were evaluated as potential enzymatic modulators of the n-3 LC-PUFA biosynthesis pathway through an *in vitro* approach with the rainbow trout liver cell line RTL-W1 enriched in ALA. The sesame seed lignans and, to a lesser extent, the enterodiol reduced the fatty acid bioconversion of the RTL-W1 cell line whereas no effect was reported for the linseed lignans. Taken as a whole, this work provides useful information in the search for feeding strategies aiming at providing the human consumers with n-3 LC-PUFA-rich fish and on the negative impact of increased water temperature that the aquaculture sector may face in the near future.

# Table of abbreviations

| | |
|---|---|
| AA | Arachidonic acid |
| ALA | α-Linolenic acid |
| BHT | Butylated hydroxytoluene |
| CD | Coefficient of distance |
| CLA | Conjugated linoleic acid |
| CoA | Coenzyme A |
| DE | Digestible energy |
| DGC | Daily growth coefficient |
| DHA | Docosahexaenoic acid |
| DM | Dry matter |
| DMSO | Dimethyl sulfoxide |
| DP | Digestible protein |
| E | Primer efficiency |
| *EF1α* | Elongation factor 1-α |
| EFA | Essential fatty acid |
| Elovl2 | Fatty acid elongase 2 enzyme |
| *elovl2* | Fatty acid elongase 2 gene |
| Elovl4 | Fatty acid elongase 4 enzyme |
| *elovl4* | Fatty acid elongase 4 gene |
| Elovl5 | Fatty acid elongase 5 enzyme |
| *elovl5* | Fatty acid elongase 5 gene |
| END | Enterodiol |
| ENL | Enterolactone |
| EPA | Eicosapentaenoic acid |
| EPI | Episesamin |
| *fads1 or FADS1* | Fatty acid desaturase 1 gene |

| | |
|---|---|
| Δ5 Fads2 | Delta 5 fatty acid desaturase |
| Δ6 Fads2 | Delta 6 fatty acid desaturase |
| *fads2 or FADS2* | Fatty acid desaturase 2 gene |
| FAME | Fatty acid methyl ester |
| Fas | Fatty acid synthase |
| FBS | Foetal bovine serum |
| FE | Feed efficiency |
| FFA | Free fatty acid |
| FHM | Fathead minnow cell line |
| GC | Gas chromatography |
| HDL | High-density lipoprotein |
| HSI | Hepato-somatic index |
| HSL | Hormone-sensitive lipase |
| L15 | Leibovitz -15 medium |
| LA | Linoleic acid |
| LC-PUFA | Long chain polyunsaturated fatty acid |
| LDL | Low-density lipoprotein |
| LER | Lipid efficiency ratio |
| LRE | Lipid retention efficiency |
| MUFA | Monounsaturated acid |
| n-3 | Omega-3 |
| n-6 | Omega-6 |
| n-9 | Omega-9 |
| NRE | Nitrogen retention efficiency |
| OLA | Oleic acid |
| PBS-EDTA | Phosphate-buffered saline supplemented with ethylenediaminetetraacetic acid |
| PCB | Polychlorinated biphenyl |
| PER | Protein efficiency ratio |

| | |
|---|---|
| PL | Phosphoglycerides |
| POP | Persistent organic pollutant |
| Ppar | Peroxisome proliferator-activated receptor |
| Pparα or PPARα | Peroxisome proliferator-activated receptor alpha |
| *pparα* | Peroxisome proliferator-activated receptor alpha gene |
| Pparβ | Peroxisome proliferator-activated receptor beta |
| *pparβ* | Peroxisome proliferator-activated receptor beta gene |
| Pparγ | Peroxisome proliferator-activated receptor gamma |
| *pparγ* | Peroxisome proliferator-activated receptor gamma gene |
| PUFA | Polyunsaturated fatty acid |
| RTL-W1 | Rainbow trout Liver-Waterloo 1 cell line |
| RT-qPCR | Quantitative reverse transcription polymerase chain reaction |
| SECO | Secoisolariciresinol |
| SES | Sesamin |
| SDG | Secoisolariciresinol diglucoside |
| SFA | Saturated fatty acid |
| SGR | Specific growth rate |
| SHK-1 | Atlantic salmon head kidney-1 cell line |
| Srebp | Sterol regulatory element binding protein |
| Srebp1c or SREBP1c | Sterol regulatory element binding protein 1c |
| *srebp1* | Sterol regulatory element binding protein 1 gene |
| Srebp2 | Sterol regulatory element binding protein 2 |
| *srebp2* | Sterol regulatory element binding protein 2 gene |
| TAG | Triacylglycerol |
| TLC | Thin-layer chromatography |
| VLDL | Very-low-density lipoprotein |
| VS | Vehicle solvent |

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# Foreword

The consumption of omega-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA, ≥ 20 carbon atoms), namely eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), has many positive effects on human health. Notably, these lipids are highly beneficial against cancer and cardiovascular diseases and are required for an optimal brain infant development and the maintenance of cognitive, inflammatory and immune functions (Ruxton *et al.*, 2005; Tocher, 2015). The dietary recommendations indicate a consumption between 250 and 500 mg per day of EPA and DHA in Belgium (CSS, 2009, 2016) and about 500 mg per day (250 mg of EPA and 250 mg of DHA) in France (ANSES, 2016). Besides their high protein, vitamin and mineral contents, fish are one of the major sources of these health-promoting fatty acids.

With the increasing awareness of n-3 LC-PUFA importance in human diet, fish consumption has been increased worldwide, especially in the developed countries (De Silva and Soto, 2009). Together with the increase of the world population, this has contributed to an increased demand for fish. However, this increased demand has emerged with the progressive depletion of wild fishery stocks and has induced a rapid development of the aquaculture sector worldwide (Hixson, 2014). To date, aquaculture provides more than half of all fish for human consumption (FAO, 2014).

In the wild, the richness of fish in n-3 LC-PUFA comes from the phytoplankton, which is eaten by the zooplankton and then by fish in case of carnivorous fish species (Pike and Jackson, 2010). In aquaculture, the high content of fish in n-3 LC-PUFA is explained by the inclusion of marine fish oil into the fish feed. However, fish oil, even more than fish meal, has become expensive and difficult to source, especially for a growing sector such as aquaculture. Moreover, given their status of finite marine resources, their use is widely criticised from a sustainable development perspective (FAO, 2014; Tocher, 2015).

There is currently an effort in feed formulation to replace, completely or partially, fish oil by alternative lipid sources. In this context, plant-derived oils are considered among the best alternatives (Turchini *et al.*, 2009; Tocher, 2015). However, unlike fish oil, plant-derived oils lack n-3 LC-

PUFA. Depending on the source, they may be rich in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), like oleic acid (OLA, 18:1n-9), and C18 polyunsaturated fatty acids (PUFA), such as linoleic acid (LA, 18:2n-6). Certain sources, such as linseed or *Camelina sativa* oils, are rich in α-linolenic acid (ALA, 18:3n-3) (Glencross, 2009; NRC, 2011). Plant-derived oils have previously been included to fish feed without compromising fish growth performance (Bell and Tocher, 2009; Turchini *et al.*, 2009). However, fish fed plant-derived oils invariably contain smaller amounts of n-3 LC-PUFA as compared to fish fed fish oil-rich diets (Thanuthong *et al.*, 2011; Francis *et al.*, 2014), resulting in major drawbacks from a human consumption perspective.

Among fish species, rainbow trout (*Oncorhynchus mykiss*) and other salmonid species possess a relatively high capacity to endogenously synthesise n-3 LC-PUFA from dietary ALA through a combination of desaturations, elongations and peroxisomal β-oxidation (Buzzi *et al.*, 1996; Sprecher, 2000; Tocher *et al.*, 2001; Tocher, 2003). When included into the feed, plant-derived oils have been reported to induce the increase of desaturation and elongation activities of salmonids (Rollin *et al.*, 2003; Thanuthong *et al.*, 2011; Turchini *et al.*, 2013; Francis *et al.*, 2014). The n-3 fatty acid bioconversion capacity of rainbow trout should therefore be maximised in order to continue providing the human consumer with fish with a significant content in EPA and DHA, while replacing fish oil by alternative lipid sources, such as the ALA-rich linseed oil, into fish feed.

Threats of climate change are numerous and varied in terms of ecosystem, society, economic and food supply issues. Among others, the aquaculture sector is at danger to be impacted by the climate change (Cochrane *et al.*, 2009; IPCC, 2014). This sector could indeed be affected directly and indirectly in the near future by pressures from, for instance, increased water temperature, reduced water supply and oxygen concentration, reduced fish meal and oil availability, increased occurrence of extreme weather events and frequency of diseases (Brander, 2007; Ficke *et al.*, 2007; De Silva and Soto, 2009). Moreover, fish being ectothermic animals, the water temperature is one of the most important environmental factors affecting their metabolism and physiology (Ficke *et al.*, 2007; Pörtner and Peck, 2010). The increased water temperature will undoubtedly affect the fatty acid metabolism of fish, especially their fatty acid bioconversion capacity. However, the extent to which this reduced capacity could affect the

development of feeding strategies based on plant-derived oils as dietary lipid sources is still poorly documented.

Even if the issues of fish oil replacement have been appreciated for many years (Naylor *et al.*, 2000), there is still a need to optimise feeding strategies in order to produce fish rich in n-3 LC-PUFA, while considering the unavoidable replacement of fish oil by economically and environmentally sustainable alternative lipid sources (Hixson, 2014; Tocher, 2015). The optimisation of fish feed, but also of fish production methods, cannot be realised without an increased knowledge of how to enhance the endogenous fatty acid bioconversion capacity of fish. A high depletion in n-3 polyunsaturated fatty acid (n-3 PUFA) of fish before the inclusion of ALA-rich oils to their feed may, for example, improve the bioconversion of ALA into n-3 LC-PUFA. The presence of bioactive compounds, such as lignans, in plant-based feed may also be one of the feeding strategies enhancing the fish n-3 LC-PUFA biosynthesis. No less importantly, the development of such feeding strategies has to be performed taking into account the emergence of new challenges, such as the increase of water temperature induced by the global warming.

Considering the above, the present work aims at evaluating the impacts of temperature, fish n-3 PUFA depletion and lignans on the fatty acid bioconversion capacity of rainbow trout, which is an important farmed fish species in cold and temperate regions (Failler *et al.*, 2007; Jalabert and Fostier, 2010). For that purpose, after reviewing the current state of knowledge (Chapter 1), this work will move further on the understanding of the effects of (1) an increased water temperature (Chapter 3), (2) a n-3 PUFA depletion of fish (Chapter 4), and (3) the addition of lignans (Chapter 5) on the fatty acid bioconversion in rainbow trout, through *in vivo* (Chapters 3 and 4) and *in vitro* (Chapter 5) approaches.

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# Chapter 1

# Literature survey

## 1.1 Rainbow trout

Rainbow trout is a fish species of the *Salmonidae* family, in the *Teleostei* infraclass. The *Oncorhynchus* genus (including rainbow trout *O. mykiss* and Pacific salmon) belongs to the *Salmoninae* subfamily with the *Salvenilus* (including Arctic char *Salvenilus alpinus* and brook trout *Salvenilus fontinalis*) and *Salmo* (including Atlantic salmon *Salmo salar* and brown trout *Salmo trutta*) genera (Jalabert and Fostier, 2010; Woynarovich *et al.*, 2011). The native range of rainbow trout is in the coastal waters and cold-tributaries of the Pacific basin, from the Kamtchatka Peninsula in Russia to the West Coast of North America. Until 1989, two populations of rainbow trout were reported, the *Salmo gairdneri* population coming from the United States and the *O. mykiss* (sometimes *Salmo mykiss*) population from the Kamtchatka peninsula. Scientific results, particularly in molecular biology, showed that both populations represented the same species and that this species had more similarities with Pacific salmon (*Oncorhynchus* sp.*)* than with Atlantic salmon *(S. salar)* (Jalabert and Fostier, 2010). Since 1870, rainbow trout has been introduced in many countries and domesticated strains are currently found in every continents, except Antarctica, as this species tolerates a wide range of environmental and production conditions as compared to other trout species (Woynarovich *et al.*, 2011; Lucas and Southgate, 2012).

In the wild, rainbow trout lives in well-oxygenated freshwater of rivers, lakes and streams. Some strains have a permanently freshwater lifetime while other anadromous strains, called steelheads, leave freshwater to stay a few years in ocean before migrating again to their freshwater birth place to spawn. Rainbow trout is considered as carnivorous but eats a wide variety of food ranging from zooplankton, crustaceans, insects and small fish. The

invertebrates represent a major part of its natural feed (Jalabert and Fostier, 2010). The natural breeding of trout populations from the United States occurs in spring between the end of March and the beginning of July in well-oxygenated cold freshwater when water temperatures reach at least 6 to 7°C. In contrast, the reproduction period of domesticated strains in Europe occurs generally from October until March when temperatures are between 10 and 15°C. Unlike the Pacific salmon, trout is iteroparous and has the ability to reproduce many times during its lifetime (Lucas and Southgate, 2012).

Rainbow trout was one of the first cultured fish species in intensive aquaculture with the use of dry formulated diets. This feeding mode had subsequently allowed the intensive farming of other fish species. Moreover, the rainbow trout farming was highly promoted by its great resistance against diseases, large dietary tolerance, easy reproduction method and fast growth (Woynarovich *et al.*, 2011). The rainbow trout farming occurs in intensive monoculture in partial or complete recirculating (raceways, ponds) or open (floating cages, tanks) systems for 8 to 14 months to provide a marketable fish. A high fish density in ponds is tolerated by rainbow trout (until 120 kg/$m^3$). The spawning is fully monitored and realised manually by abdominal pressure on a slightly anaesthetised fish (Lovell, 2003; Lucas and Southgate, 2012).

Although the rainbow trout and other salmonid farming represents a small part of the global aquaculture, the rainbow trout farming is particularly developed in Europe (Failler *et al.*, 2007; Shepherd *et al.*, 2016). Moreover, within the salmonid aquaculture, rainbow trout and Atlantic salmon are among the most commonly cultured species worldwide (Lovell, 2003; Failler *et al.*, 2007; Lucas and Southgate, 2012). In 2014, the rainbow trout farming represented 1.1 % of the total world production with 810 000 tonnes of fish whereas the Atlantic salmon production amounted to 3.2 % with 2 300 000 tonnes (FAO, 2014b; Marine Harvest, 2016). In Europe, the total production of rainbow trout amounted 260 000 tonnes in 2012 (FAO, 2012). The major salmonid-producing countries are Scotland, France, Denmark, Spain and Germany within the European Union, and Chile, Norway, Turkey and the United States worldwide (Lucas and Southgate, 2012; FAO, 2014b). The aquaculture production in Belgium, and in Wallonia, is reduced and mainly focused on rainbow trout small-scale farming. In Belgium, the production of rainbow trout was estimated at 175 tonnes in 2014. Rainbow trout is one of the most cultivated species in Wallonia. Indeed, 79 % of fish

farmed in Wallonia in 2012 belong to the trout species (*O. mykiss*, *S. trutta* and *S. fontinalis*) (CFWP, 2016; SoCoPro, 2016). The other fish species farmed in Wallonia in 8 % of sturgeons and 13 % of other species (cyprinids) (CFWP, 2016).

*Nutrient and energy requirements*

A dietary compound is essential when fish cannot synthesise it on its own in sufficient quantities for its optimal growth and development. Rainbow trout is the fish species for which the dietary requirements have been the most accurately determined. The dietary requirements in nutrients and energy of rainbow trout are summarised in Table 1.1.

Fish require approximately a two-fold higher dietary protein level as compared to other animals. The recommended dietary protein level of rainbow trout is about 38 % of the dry matter (DM) for a fish ranging between 200 g and 1 500 g. The requirement level is higher for smaller fish (from 40 to 48 %) and lower for bigger fish (36 %) (NRC, 2011). The digestible protein to digestible energy (DP/DE) ratio is another way of expressing protein requirement. For species accepting high dietary lipid levels, such as rainbow trout, the optimum DP/DE ratio is around 20 g of DP per MJ of DE (NRC, 2011). Moreover, an optimal dietary amino acid profile is also required for the efficient growth and development of fish. As other vertebrates, 10 amino acids are considered as essential for fish, these include arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The dietary amino acid requirements of rainbow trout have been previously reported (NRC, 2011) and are presented in Table 1.1. In fish feed formulation, fish meal is the protein source with the most appropriate amino acid profile (Guillaume *et al.*, 2001).

Defining the dietary requirement of fish for fatty acids is depending on the functional role of the fatty acids and the fish ability to endogenously produce them. Considering salmonids, LA, ALA, EPA and DHA are considered as essential fatty acids (EFA) (NRC, 2011). In contrast, the requirements for n-6 LC-PUFA in freshwater fish have still not been determined (NRC, 2011). The juvenile and sub-adult rainbow trout requirements can be covered if the diet contains around 1 % of LA and 1 % of ALA of the diet DM. However, being coldwater species, salmonids possibly have a higher requirement for ALA as compared to LA (NRC, 2011). The fish requirements for n-3 PUFA can also be covered by supplying fish with 0.5 % of EPA + DHA in the diet

DM (NRC, 2011). The requirement for EFA differs with the fish ontogeny and probably also with the dietary lipid content (Tocher, 2010). Tocher (2010) reported that the requirement in n-3 LC-PUFA, particularly DHA, may be higher at early life stages, as compared to the juvenile and adult fish requirements. A deficiency in EFA induces severe deleterious effects in fish, such as impaired growth on a short term, but also hepatic deterioration, gill and skin damages, impaired vision, reduced fecundity, reduced embryonic viability and decreased survival at early life stage when fish is exposed to a longer deficiency period (Castell *et al.*, 1972; Glencross, 2009). The appropriate feed content for a normal growth of adult salmonids is about 20-30 % of fatty acids in dry feed (NRC, 2011; Lucas and Southgate, 2012). However, salmonids have no requirement for dietary lipid level *per se*, once the dietary fatty acid requirements are supplied. Considering dietary phospholipids, a quantitative requirement of 4 to 14 % of diet weight in phospholipids has been reported for optimal growth of rainbow trout juvenile (Poston, 1990; Rinchard *et al.*, 2007), underlying the reduced capacity for phospholipids biosynthesis in enterocytes of salmonids at the early developmental stage (Carmona-Antoñanzas *et al.*, 2015). In the wild, the EFA are mainly found in the phytoplankton, rich in LA and ALA in freshwaters or in EPA and DHA in marine waters, and are efficiently retained at higher trophic levels. In fish feed formulation, fish oil is the dietary lipid source containing the highest level of EPA and DHA (Guillaume *et al.*, 2001).

No requirement for dietary carbohydrates has been reported so far for rainbow trout (NRC, 2011). However, if present in the fish feed, carbohydrates spare dietary proteins and lipids as sources of energy. In the natural feed of rainbow trout, carbohydrates usually represent a small part of the diet (Guillaume *et al.*, 2001).

Considering minerals and vitamins, the fish requirements are easily covered by artificial feed. Indeed, in fish feed formulation, the mineral source can be the feed components themselves or a premix incorporated into the feed, whereas vitamins are primarily added as premix in order to compensate for the complexity to quantify the vitamin concentration in feed components (NRC, 2011). The dietary mineral and vitamin requirements of rainbow trout are reported in Table 1.1. In addition, the carotenes and carotenoids, such as β-carotene and astaxanthin, have to be supplied through the feed in order to induce the typical pigmentation of rainbow trout skin and fillet, which is a

quality criterion for consumers (Guillaume *et al.*, 2001; Lovell, 2003; NRC, 2011).

The energy requirement of fish is relatively low as fish are ectothermic animals, the body temperature varying according to the water temperature (Bureau *et al.*, 2003). For an optimal growth, rainbow trout feed must contain about 17.6 MJ of digestible energy (DE) per kg of dry feed (NRC, 2011). In contrast with mammals, dietary proteins and lipids are the major sources of energy for carnivorous fish, whereas carbohydrates represent a minor source (NRC, 2011). The basal metabolism of rainbow trout is estimated from 29.29 to 37.66 kJ per kg of body weight$^{0.80}$ per day for fish maintained between 15 and 18°C (NRC, 2011). However, the basal metabolism is related to the fish size, since it increases, in absolute terms (kJ per animal per day), with the animal mass increase, as well as with the water temperature, which has a determinant effect on biochemical reactions and metabolic rates (Bureau *et al.*, 2003). The maintenance energy requirement is higher than the basal metabolism and is estimated for rainbow trout from 41.84 to 71.13 kJ of metabolisable energy per kg of body weight$^{0.80}$ per day for fish of about 100 g at a water temperature of 14 to 18°C (NRC, 2011).

**Table 1.1.** Nutrient and energy requirements of rainbow trout

| **Digestible protein level (% dry diet)** | 38 |
|---|---|
| **Digestible energy (MJ/kg)** | 17.6 |
| **Amino acids (% dry diet)** | |
| Arginine | 1.5 |
| Histidine | 0.8 |
| Isoleucine | 1.1 |
| Leucine | 1.5 |
| Lysine | 2.4 |
| Methionine | 0.7 |
| Methionine + cysteine | 1.1 |
| Phenylalanine | 0.9 |
| Phenylalanine + tyrosine | 1.8 |
| Threonine | 1.1 |
| Tryptophan | 0.3 |
| Valine | 1.2 |

**Table 1.1** - Continued

| | |
|---|---|
| **Fatty acids (% dry diet)** | |
| LA | 1 |
| ALA | 1 |
| n-3 LC-PUFA | 0.5 |
| **Macrominerals (% dry diet)** | |
| Phosphorus | 0.7 |
| Magnesium | 0.05 |
| **Microminerals (mg/kg dry diet)** | |
| Copper | 3 |
| Zinc | 15 |
| Manganese | 12 |
| Selenium | 0.15 |
| **Water-soluble vitamins (mg/kg dry diet)** | |
| Thiamin | 1 |
| Riboflavin | 4 |
| B6 | 3 |
| Pantothenic acid | 20 |
| Niacin | 10 |
| Biotin | 0.15 |
| B12 | R |
| Folate | 1 |
| Choline | 800 |
| Myoinositol | 300 |
| C | 20 |
| **Lipid-soluble vitamins (units/kg dry diet)** | |
| A | 0.75 mg |
| D | 40 µg |
| E | 50 mg |
| K | R |

These requirements have been determined with highly purified ingredients in which the nutrients are highly digestible; therefore the values presented represent near 100 % bioavailability. LA, linoleic acid; ALA, α-linolenic acid; n-3 LC-PUFA, n-3 long chain polyunsaturated fatty acids, R: required but no value determined. Adapted from NRC (2011).

*Physical and environmental requirements*

Fish are poikilothermic animals, their body temperature varying with the water temperature and ectothermic animals, the body heat coming principally from water. Moreover, trout is a stenothermal species tolerating only limited fluctuations around its optimal growth temperature, as compared to eurythermal organisms. Trout is therefore doubly dependent on water temperature, which is thus an important environmental parameter for its optimal development and growth. Although being a stenothermic animal, rainbow trout has an incipient lethal temperature range from 0 to 24°C (Myrick and Cech Jr, 2000; De Silva and Soto, 2009; Lucas and Southgate, 2012), the upper limit varying with fish size, fish strain and the dissolved oxygen level in water. Lacking antifreeze proteins, salmonids cannot tolerate temperatures below 0°C (Jalabert and Fostier, 2010). It is worth noting that a sudden shift of water temperature may lead to death, even within the temperature range mentioned above. The fish growth is stopped under 4°C and is slowed down around 20°C, even though trout tolerates these temperatures (Bear *et al.*, 2007; De Silva and Soto, 2009; Jalabert and Fostier, 2010). Salmonids appears to have a very similar thermal tolerance, irrespective of the strain or the origin, such as observed by Myrick and Cech (2000) who have compared the rainbow trout strain from the Eagle lake (*O. m. aquilarum*) to the Mt. Shasta strain. The optimal temperature range for growth and spawning of rainbow trout is between 9 and 16°C (Bear *et al.*, 2007; Ficke *et al.*, 2007; De Silva and Soto, 2009; Woynarovich *et al.*, 2011; Lucas and Southgate, 2012). Jalabert and Fostier (2010) reported an optimal growth temperature of 15-16°C for the juvenile and adult rainbow trout. In contrast, optimal spawning temperatures range from 10 to 13°C. A temperature from 10 to 12°C is required during the maturation of the broodstock fish and the egg incubation in order to obtain a good fish quality (Woynarovich *et al.*, 2011; Lucas and Southgate, 2012).

Rainbow trout requires a dissolved oxygen concentration of minimum 5 mg/l of water, with >70 % of saturation. Below this limit, fish are under hypoxia and their metabolism is negatively impacted (Jalabert and Fostier, 2010; Lucas and Southgate, 2012). An increased level of dissolved gaseous nitrogen to above 110 % of saturation causes gas bubble trauma. The concentration in carbon dioxide is tolerated at maximum 10 mg/l, the ammonia ($NH_3$) has to be below 0.0125 mg/l, the nitrites ($NO_2^-$) below 0.1 mg/l and the pH between 5.5 and 8.5 (Lucas and Southgate, 2012).

## 1.2 Fatty acid metabolism in salmonids

### 1.2.1 Fatty acid structure and functions

Fatty acids are organic compounds composed by an acid function and an aliphatic chain of 4 to 36 carbon atoms (Lehninger *et al.*, 2008). In addition to the trivial name, fatty acids are also termed X:Yn-Z, where X is the number of carbon atoms of the aliphatic chain, Y is the unsaturation degree (number of ethylenic/double bonds), and n-Z is the position of the first double bond from the methyl end of the aliphatic chain, as set by the International Union of Pure and Applied Chemists (IUPAC). They can be classified into three groups depending on the unsaturation degree of the aliphatic chain: the SFA hold a chain without ethylenic bond whereas MUFA own one ethylenic bond and PUFA own two or more ethylenic bonds, generally separated by a single methylene group. LC-PUFA, a subgroup of PUFA, has an aliphatic chain equal or longer than 20 carbons with at least four ethylenic bonds (Tocher, 2003; Glencross, 2009; NRC, 2011). The position of the first ethylenic bond at 3, 6, or 9 carbon atoms from the methyl end of the fatty acid defines the n-3, n-6 and n-9 PUFA families, respectively. The predominant SFA present in wild salmonids are palmitic acid (16:0) and stearic acid (18:0), the predominant MUFA are OLA, gadoleic acid (20:1n-11) and cetoleic acid (22:1n-11) and the predominant PUFA are LA, arachidonic acid (AA, 20:4n-6), EPA and DHA (Tocher, 2003; Henriques *et al.*, 2014). The palmitic acid, OLA, AA, EPA and DHA are presented in Table 1.2.

**Table 1.2**. Nomenclature of some naturally occurring fatty acids in wild salmonids

| Carbon skeleton | Systematic name | Common name | Abbreviation |
|---|---|---|---|
| 16:0 | n-Hexadecanoic acid | Palmitic acid | - |
| 18:1n-9 | cis-9-Octadecenoic acid | Oleic acid | OLA |
| 20:4n-6 | cis-,cis-,cis-,cis-5, 8, 11, 14-Eicosatetraenoic acid | Arachidonic acid | AA |
| 20:5n-3 | cis-,cis-,cis-,cis-,cis-5, 8, 11, 14, 17- Eicosapentaenoic acid | Eicosapentaenoic acid | EPA |
| 22:6n-3 | cis-,cis-,cis-,cis-,cis-,cis-4, 7, 10, 13, 16, 19- Docosahexaenoic | Docosahexaenoic acid | DHA |

In animals, fatty acids are present as free fatty acids (FFA). The major part is however included in triacylglycerols (TAG), which are neutral lipids consisting of three fatty acids esterified to the hydroxyl groups in *sn*1, *sn*2 and *sn*3 positions of a glycerol moiety. In addition, fatty acids are also included in phosphoglycerides (PL), which are polar lipids including two fatty acids esterified to the hydroxyl groups in *sn*1 and *sn*2 positions of a glycerol moiety linked to a polar head group, as well as in sphingolipids, which are polar lipids consisting in a single fatty acid linked through an amide link to a sphingosine moiety. In TAG of fish, SFA and MUFA are generally located in the *sn*1 and *sn*3 positions, whereas PUFA are generally located in the *sn*2 position. Similarly, the PL are generally composed of SFA and MUFA in *sn*1 position and of PUFA in *sn*2 position (Tocher, 2003; Lehninger *et al.*, 2008; Tocher *et al.*, 2008). Zooplankton and some fish species (such as marine myctophid fishes) may also contain significant amounts of wax esters, which are neutral lipids composed one fatty acid esterified to one fatty alcohol (Tocher, 2003).

Lipids, in particular fatty acids, play significant roles such as cellular energy supply, involvement in cell membrane formation and functions, and signalling molecule synthesis. In sufficient dietary concentration to cover the fatty acid requirements, fatty acids, as TAG, are a major energy source used by fish for growth, swimming and reproduction. The excess dietary energy is stored and may subsequently expend through β-oxidation during migration or starvation. The extent to which any fatty acid is oxidised to produce energy depends on the fatty acid structure and its dietary concentration, with the exception of DHA, which is mostly retained independently of its dietary concentration (Tocher, 2003; NRC, 2011).

As PL and sphingolipids are fundamental elements of the lipid bilayer, the fatty acids have a significant impact on the cellular membrane composition and functions. The amphipathic structure of PL, having both hydrophilic (polar head group) and hydrophobic (glycerol, *sn*1 and *sn*2 fatty acids) groups, and the adaptation in fatty acid composition of PL, allow a dynamic change of cellular membranes and therefore of cell metabolism. SFA ensure membrane rigidity while MUFA and PUFA bring more fluidity thanks to their higher unsaturation degree. Fish being ectothermic animals, the membrane fluidity through the PUFA composition adaptation is essential for the fish survival in a seasonal changing environment (Farkas *et al.*, 2001). Moreover, the strong but flexible structure of DHA allows a rapid cell

membrane reorganisation and explains the high concentration of DHA in brain, retinal and synaptic membranes (Glencross, 2009). The cellular membrane fatty acids also influence the functions of membrane proteins (receptors, carriers and enzymes) (Spector and Yorek, 1985; Stubhaug *et al.*, 2005; Lehninger *et al.*, 2008).

PUFA composed of 20 carbons, namely AA and EPA, are precursors for the synthesis of highly bioactive paracrine hormones called eicosanoids. These compounds, which are produced in most tissues, are involved in cellular functions such as immune and inflammatory responses, renal and neural functions, blood clotting and reproduction (Tocher, 2003; Lehninger *et al.*, 2008; Schmitz and Ecker, 2008). The synthesis of eicosanoids is catalysed by cyclooxygenases and lipoxygenases to form either cyclic oxygenated derivatives (prostaglandins, prostacyclins and thromboxanes) or linear oxygenated derivatives (hydroperoxy- and hydroxyl fatty acids, leukotrienes and lipoxins) (Tocher, 2003). The eicosanoids derived from n-6 PUFA highlight differential or even sometimes contradictory responses to those derived from n-3 PUFA. As an example, n-6 PUFA-derived eicosanoids usually exhibit a pro-inflammatory response, whereas their n-3 PUFA counterparts present less pro-inflammatory or even anti-inflammatory responses (Schmitz and Ecker, 2008; Wall *et al.*, 2010). The EPA and DHA can also be metabolised into resolvins and protectins, which are endogenous local mediators possessing potent anti-inflammatory and immunoregulatory properties (Wall *et al.*, 2010).

PUFA have also a key role in regulating the expression of genes involved in cellular lipid homeostasis through their action on transcription factors. This point is detailed in Section 1.3.2.1 Transcription factors.

It ensues from the numerous functions of fatty acids at a cellular level described above that fatty acids have significant effects on fish growth and diverse physiological processes, such as resistance to stress and disease, immune function, neuronal development and visual function. Moreover, as regards the n-3 PUFA, ALA has relatively little functional role, apart from being the precursor of EPA and DHA (Tocher, 2003; Glencross, 2009; Tocher, 2015).

### 1.2.2 Fatty acid digestion and absorption

The dietary lipids of fish feed are mainly composed of TAG, as well as of PL, sphingolipids, sterols, free fatty acids, pigments and waxes as minor components (Glencross, 2009; Bakke *et al.*, 2011). The lipid assimilation begins with two different steps: the digestion of lipids by enzymatic hydrolysis and their uptake by enterocytes.

The buccal and stomach lipid digestions are respectively absent and reduced in fish. The ingested lipids are therefore mainly digested in the proximal and distal parts of the intestine and the pyloric caeca (finger-like pouches of the intestine) (Guillaume *et al.*, 2001; Glencross, 2009).

Lipases are involved in the fish lipid digestion and are secreted by the pancreas and the intestinal mucosa. Different forms of lipases exist in rainbow trout: the lipase-colipase system and, probably, to a lesser extent, other biliary salt-activated lipases cleave TAG, phospholipases cleave PL and other esterases are dedicated to cholesteryl esters (Leger *et al.*, 1977; Tocher and Sargent, 1984; Tocher *et al.*, 2008; Kurtovic *et al.*, 2009; Bakke *et al.*, 2011). These cleavages generate mainly free fatty acids, but also monoacylglycerols, diacylglycerols, lysophospholipids and cholesterol. No lipases cleaving the *sn*2 position of the TAG and PL have been identified in fish. Since most of the LC-PUFA are found in the *sn*2 position of TAG and PL, it is assumed that a major part of the dietary LC-PUFA is absorbed by enterocytes as monoacylglycerols, diacylglycerols, and lysophospholipids (Glencross, 2009).

Lipids are difficult to digest due to their hydrophobic nature and the hydrophilic nature of the enzymes. The enzymatic hydrolysis is therefore facilited by the emulsification of lipids, which increases the surface contact between the substrates and enzymes. The biliary emulsifiers, such as taurocholate, are produced by the liver and stored in the gallbladder. They support the formation of emulsions, which facilitate the lipase action on lipids, and of mixed-micelles, which increase the uptake of fatty acids and other digested products by enterocytes (Tocher, 2003; Glencross, 2009; Bakke *et al.*, 2011).

The lipid absorption principally occurs in the pyloric caeca and the mid-intestine, as observed in Atlantic salmon (Denstadli *et al.*, 2004). The facilitated transport and the passive diffusion through the intestinal barrier

are the absorption mechanisms, probably occurring respectively at low and high luminal fatty acid concentration (Tso *et al.*, 2004; Bakke *et al.*, 2011). However, the presence of transporters in enterocytes membranes of fish has still to be elucidated (Bakke *et al.*, 2011). According to Oxley *et al.* (2005), OLA, LA and DHA have a higher absorption rate by rainbow trout enterocytes as compared to ALA, 20:1n-9, AA and EPA. In enterocytes, most of free fatty acids and other digested products are re-esterified into TAG and PL. Lipids which are not re-esterified (particularly the < 12C SFA) are catabolised by enterocytes, or directly absorbed into the circulation and either catabolised later by β-oxidation or stored in fish tissues (Glencross, 2009).

In fish, the lipid digestion and absorption are influenced by a range of different factors, such as the fish species, the fish developmental stage, the water temperature, the dietary fatty acid length and its degree of unsaturation (Glencross, 2009; Bakke *et al.*, 2011). The digestibility of lipids is quantified through the apparent digestibility coefficient, which is determined relative to the dietary and faecal concentrations of the compound of interest and of an indigestible marker (Guillaume *et al.*, 2001; Bureau *et al.*, 2003). As compared to other dietary constituents, fatty acids have a high digestibility of over 85 %, irrespective of the dietary animal or plant origin (Sigurgisladottir *et al.*, 1992; Ng *et al.*, 2010). The digestibility of a fatty acid is decreased with increasing aliphatic chain length and increased with increasing unsaturation degree. The longer the aliphatic chain, the lower the digestibility (Austreng *et al.*, 1980; Sigurgisladottir *et al.*, 1992; Ng *et al.*, 2003; Ng *et al.*, 2010). On the other hand, PUFA, because of their higher unsaturation degree, present a higher apparent digestibility, as compared to SFA and MUFA (Austreng *et al.*, 1980; Caballero *et al.*, 2002; Ng *et al.*, 2003; Francis *et al.*, 2007; Ng *et al.*, 2010). The apparent digestibility is also decreased as the position of the first double bond moved further the methyl end of the aliphatic chain (n-3 > n-6 > n-9) (Francis *et al.*, 2007). The fatty acid digestibility is also influenced by the water temperature, the dietary lipid source and the dietary n-3/n-6 ratio (Grisdale-Helland *et al.*, 2002; Ng *et al.*, 2010; Huguet *et al.*, 2015). This will be detailed in Sections 1.3.2.1 and 1.4.2.

### 1.2.3 Fatty acid transport

After digestion and absorption, lipids are transported up to the tissues by the lymphatic system and the systemic circulation. The lipid transport mainly

occurs through the use of lipoproteins, which allow the transport of hydrophobic compounds in the aqueous plasma medium. Lipoproteins are macromolecule structures containing lipids and apolipoproteins. Their surface layer is made by apolipoproteins and the hydrophilic head groups of PL, which ensure cohesion and solubility in blood and lymph, and the hydrophobic lipids at the core. Lipoproteins are classified according to their density and size. The chylomicrons, which export lipids from enterocytes to hepatocytes and other tissues, are the lipoproteins with the lowest density. The very-low-density lipoproteins (VLDL) also export lipids from intestine to liver, but the majority of VLDL is synthesised in hepatocytes in order to supply the fish tissues in lipids from hepatocytes. The low-density lipoproteins (LDL) derive from the VLDL through neutral lipid delivery to the extra-hepatic tissues. Their role is to provide cholesterol to the extra-hepatic tissues. In contrast, the high-density lipoproteins (HDL) take up cholesterol from the extra-hepatic tissues to return to the liver. In trout, HDL is the predominant lipoprotein class, followed by LDL and then VLDL (Babin, 1987; Babin and Vernier, 1989; Tocher, 2003). In rainbow trout, Babin and Vernier (1989) reported that chylomicrons, VLDL, LDL and HDL were composed of 84 %, 52 %, 22 % and 11 % of TAG and of 8 %, 19 %, 27 % and 32 % of PL, respectively. After hydrolysis of the TAG present in lipoproteins by the extracellular lipoprotein lipases, the fatty acids are taken up by cells through a passive mechanism or by plasma membrane proteins (Stubhaug *et al.*, 2005; Tocher *et al.*, 2008). The involvement of membrane bound fatty acid uptake proteins, namely fatty acid transport protein and fatty acid translocase, has been reported in liver, fillet and adipose tissue (Torstensen *et al.*, 2009) and in cultured primary hepatocytes (Zhou *et al.*, 2010) of Atlantic salmon. In hepatocytes, different metabolic pathways occur such as lipogenesis, fatty acid β-oxidation, elongation and desaturation. Fatty acids can be included as TAG and PL in newly synthesised lipoproteins, which will be then secreted by hepatocytes (Tocher, 2003; Glencross, 2009).

### 1.2.4 Fatty acid biosynthesis and bioconversion

In addition to the impact of the composition of dietary lipids, the fatty acid composition of salmonids can be modulated through different metabolic processes occurring mainly in hepatocytes. Some fatty acids are synthesised *de novo* while others are endogenously produced from dietary precursors, such as LA and ALA.

#### 1.2.4.1 Neosynthesis

The fatty acid neosynthesis is the term used to describe the endogenous production of new SFA. The process starts with the production of palmitic acid (16:0) and mainly occurs in the cytosol of hepatocytes, and to a lesser extent, in that of adipocytes in the case of rainbow trout (Lin *et al.*, 1977). Acetyl-CoA is the carbon source for this biosynthesis and is produced in mitochondria from the degradation of amino acids (protein source) and, to a lesser extent, of pyruvate (carbohydrate source). The pathway starts by the condensation of an acetyl-CoA with a malonyl-CoA, which is previously formed from acetyl-CoA by the acetyl-CoA carboxylase (Lehninger *et al.*, 2008; Castro *et al.*, 2016). The aliphatic chain is subsequently lengthened by sequential condensation steps of two carbon units through malonyl-CoA. Every addition of two carbon atoms proceeds in four steps (condensation, reduction, dehydration and a second reduction) requiring NADPH and is catalysed by the cytosolic fatty acid synthase (Fas), which is a polypeptide bearing seven enzymatic activities. The SFA 16:0 and 18:0 are the main products of this cytosolic process (Tocher, 2003; Castro *et al.*, 2016). Longer SFA (up to 24 carbon atoms) are synthesised in both mitochondria and endoplasmic reticulum following a similar sequential process but involving other enzymes (Lehninger *et al.*, 2008; Castro *et al.*, 2016).

#### 1.2.4.2 Monounsaturated fatty acid biosynthesis and bioconversion

Fish have the capacity to biosynthesise MUFA *de novo* in the endoplasmic reticulum. The 16:1n-7 and OLA are produced respectively from 16:0 and 18:0 thanks to the Δ9 desaturase, also called stearoyl CoA desaturase, which introduces a double bond between the carbon atoms 9 and 10 from the carboxyl end of the fatty acid. The fatty acyl-CoA is the substrate of this oxygen and NADPH-dependent enzyme (Nakamura and Nara, 2004). The Δ9 desaturase is also described as a mixed-function oxidase since saturated fatty acyl-CoA and NADPH are both oxidised by molecular oxygen (Lehninger *et al.*, 2008). Once synthesised, the MUFA can be processed into PUFA of the n-9 family through the activity of Δ6 and Δ5 desaturases and elongases (Tocher, 2003).

### 1.2.4.3 Polyunsaturated fatty acid bioconversion

#### *1.2.4.3.1 Metabolic pathway*

LA and ALA are, respectively, the precursor compounds of the n-6 and n-3 PUFA families and are considered as essentials for vertebrates, which cannot synthesise them *de novo*. In contrast, unlike animals, plants possess specific enzymes, the Δ12 and Δ15 desaturases, to synthesise, respectively, LA and ALA from OLA (Tocher *et al.*, 1998). The metabolic pathways involved in the bioconversion of the n-6 and n-3 precursor LA and ALA into their more highly unsaturated LC-PUFA are schematically represented in Figure 1.1.

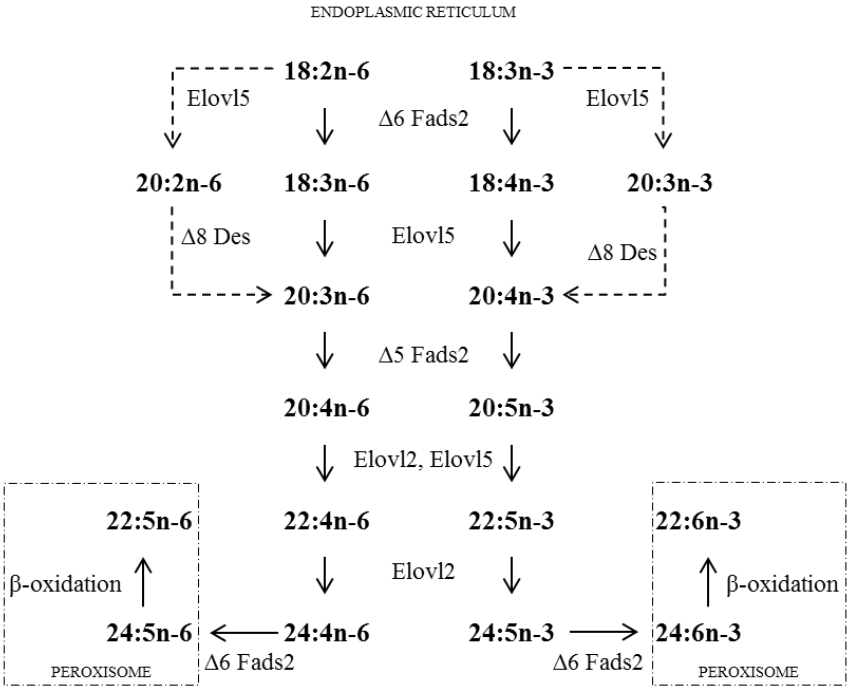


**Figure 1.1.** Synthesis of n-3 and n-6 long chain polyunsaturated fatty acids in salmonids. Δ5 Fads2, Δ5 fatty acid desaturase; Δ6 Fads2, Δ6 fatty acid desaturase; Δ8 Des, Δ8 desaturase; Elovl2, very long fatty acid elongase 2; Elovl5, very long fatty acid elongase 5; 18:2n-6, linoleic acid; 18:3n-6, γ-linolenic acid; 20:2n-6, eicosadienoic acid; 20:3n-6, dihomo-γ-linolenic acid; 20:4n-6, arachidonic acid; 22:4n-6, docosatetraenoic acid; 24:4n-6, tetracosatetraenoic acid; 24:5n-6, tetracosapentaenoic acid; 22:5n-6, docosapentaenoic acid; 18:3n-3, α-linolenic acid; 18:4n-3, stearidonic acid; 20:3n-3, eicosatrienoic acid; 20:4n-3, eicosatetraenoic acid; 20:5n-3, eicosapentaenoic acid; 22:5n-3, docosapentaenoic acid; 24:5n-3, tetracosapentaenoic acid; 24:6n-3, tetracosahexaenoic acid; 22:6n-3, docosahexaenoic acid. Adapted from Tocher (2015).

The bioconversion of n-3 and n-6 fatty acid precursors occurs in the endoplasmic reticulum of hepatocytes and enterocytes with the substrates and products as acyl-CoA derivatives (Sprecher, 2000). ALA is alternately desaturated and elongated to produce EPA and DHA. Similarly, LA is bioconverted into AA. DHA and AA are respectively the main end-products of the n-3 and n-6 metabolic pathways, although AA can be further desaturated and elongated. The bioconversion of EPA into DHA is reported as the Sprecher pathway and involves the elongation of EPA into 22:5n-3 and 24:5n-3, the Δ6 desaturation into 24:6n-3 and finally the translocation of 24:6n-3 to peroxisomes in order to shorten the aliphatic chain via β-oxidation to produce DHA (Buzzi *et al.*, 1996, 1997; Sprecher, 2000). The bioconversion of AA in 22:5n-6 is following the same pathway. These reactions are catalysed by desaturases and elongases, which have the capacity to work with substrates from each fatty acid family (Buzzi *et al.*, 1996; Tocher, 2003). In parallel to the n-6 biosynthesis pathway, LA is also elongated through the "dead end" elongation pathway in 20:2n-6 (which can then be Δ8 desaturated in 20:3n-6), 22:2n-6 and 24:2n-6. Similarly, ALA can be elongated in 20:3n-3 (and then Δ8 desaturated in 20:4n-3), 22:3n-3 and 24:3n-3 (Buzzi *et al.*, 1996; Monroig *et al.*, 2011).

All fish possess the enzymatic system to metabolise the dietary LA and ALA into LC-PUFA. However, the fish bioconversion capacity highly depends on both the fish species and its environment. Unlike marine fish species, some freshwater and anadromous fish, such as salmonids, are known to successfully biosynthesise LC-PUFA. The difference of efficiency between marine and freshwater fish is often explained by the phytoplankton in marine environment, which is rich in n-3 LC-PUFA, whereas the phytoplankton in freshwater is characterised by higher amounts in C18 PUFA, LA and ALA. Beyond the habitat aspect, the trophic level, the "trophic ecology" and diadromy also modulate the fish LC-PUFA biosynthesis capacity (Castro *et al.*, 2016). This has led marine fish to rely, to a lesser extent, on their endogenous fatty acid bioconversion to meet their essential fatty acid requirements, and therefore to a down-regulation of the activity of the enzymes and expression of their associated genes (Tocher, 2003, 2010; Morais *et al.*, 2012; Tocher, 2015; Castro *et al.*, 2016).

#### *1.2.4.3.2 Desaturases*

Two acyl-CoA desaturases are involved in the n-9, n-6 and n-3 bioconversion pathways, namely the Δ5 and Δ6 fatty acid desaturases (Fads2). In mammals, fatty acid desaturase 1 (*FADS1*) is the gene encoding the Δ5 Fads2 and fatty acid desaturase 2 (*FADS2*) the Δ6 Fads2 (Marquardt *et al.*, 2000). In salmonids and other teleost fish, the *fads1* gene has been lost and only *fads2* genes have been cloned and reported to encode for desaturases with a Δ5 or Δ6 enzymatic activity (Seiliez *et al.*, 2001; Hastings *et al.*, 2004; Zheng *et al.*, 2004a; Monroig *et al.*, 2010; Castro *et al.*, 2016; Hamid *et al.*, 2016). For instance, Atlantic salmon possess four *fads2* genes that encode proteins with Δ5 (one protein) or Δ6 (three proteins) desaturase activities (Monroig *et al.*, 2010). The reported *fads2* genes in salmonids have a high homology with the human *FADS2* when comparing the sequences (Zheng *et al.*, 2005a). In rainbow trout, the highest levels of expression of *fads2* have been found in liver, intestine and brain (Seiliez *et al.*, 2001; Hamid *et al.*, 2016), whereas no expression was reported for the stomach, fillet and adipose tissue (Hamid *et al.*, 2016). In Atlantic salmon, the liver, intestine and brain presented also the highest level of expression (Zheng *et al.*, 2005a; Monroig *et al.*, 2010).

The Δ5 and Δ6 Fads2 are transmembrane proteins of the endoplasmic reticulum present in different tissues of rainbow trout such as liver, brain, intestine and kidney (Seiliez *et al.*, 2001). A fatty acyl-CoA Fads2 with a Δ6 enzymatic activity was cloned and characterised in rainbow trout by Seiliez *et al.* (2001) and subsequently by Zheng *et al.* (2004a). The open reading frame of the trout desaturase-like cDNA reported by Seiliez *et al.* (2001) encodes a 454-amino acid peptide containing three histidine boxes and two membrane-spanning domains. The Δ6 Fads2 also contains a N-terminal cytochrome b5-like domain containing the heme-binding motif HPGG, which is presumably involved in the electron transport chain required for the acyl-desaturation (Seiliez *et al.*, 2001; Venegas-Calerón *et al.*, 2010; Vagner and Santigosa, 2011). A Δ5 Fads2 was firstly isolated and functionally characterised in Atlantic salmon (Hastings *et al.*, 2004) and has also been recently isolated in rainbow trout (Hamid *et al.*, 2016). The 454-amino acid protein of rainbow trout also included a N-terminal cytochrome b5-like domain containing the heme-binding motif HPPG, three histidine boxes and three membrane-spanning regions and was highly similar to the Δ6 Fads2 (Hamid *et al.*, 2016). The reported regions in Δ5 and Δ6 Fads2 of rainbow

trout are perfectly aligned with those of the Δ5 and Δ6 desaturases of human and other vertebrates, with the exception of ten additional amino acid residues at the N-terminal end (Seiliez *et al.*, 2001; Zheng *et al.*, 2004a), as also observed for Atlantic salmon (Hastings *et al.*, 2004; Zheng *et al.*, 2005a; Monroig *et al.*, 2010). Interestingly enough, our group has been recently associated to a study on the cloning, tissue expression and functional characterisation of the Eurasian perch (*Perca fluviatilis*) *fads2* (Geay *et al.*, 2016). The *fads2* cDNA encoded a protein of 445 amino acids displaying a N-terminal cytochrome b5-like domain containing the heme-binding motif HPPG and two transmembrane domains. The *fads2* cDNA was mainly expressed in the liver and intestine and coded for a desaturase with a fully functional Δ6 desaturation and residual Δ5 desaturation activities (Geay *et al.*, 2016).

Both Δ5 and Δ6 Fads2 have the capacity to introduce an additional double bond into the aliphatic chain of fatty acids, respectively, at 5 or 6 carbon atoms from the carboxyl end of the molecule. Despite the high similarity of amino acid sequences of Δ5 and Δ6 Fads2, the enzymes have distinct enzymatic activities (Monroig *et al.*, 2010). The Δ5 Fads2 serves in only one step in order to desaturate 20:3n-6 and 20:4n-3. In contrast, the Δ6 Fads2 can operate on both LA and ALA in a first step and on 24:4n-6 and 24:5n-3 in a second step of the metabolic pathway (Figure 1.1) (Buzzi *et al.*, 1996; Tocher, 2003; Vagner and Santigosa, 2011). In fish species, it is still not clear if the same Δ6 Fads2 catalyses both steps or if isoenzymes could exist (Nakamura and Nara, 2004; Vagner and Santigosa, 2011). However, in human, the same enzyme acts on both steps (de Antueno *et al.*, 2001). Interestingly, both salmonid Δ5 and Δ6 Fads2 have also a minor capacity to insert a double bond in Δ6 and Δ5 positions, respectively (Hastings *et al.*, 2004; Zheng *et al.*, 2004a; 2005a). Conversely to salmonids, only one protein having both the Δ5 and Δ6 activities exists in zebrafish (Hastings *et al.*, 2001), rabbitfish (*Siganus canaliculatus*) (Li *et al.*, 2010), pike silverside (*Chirostoma estor*) (Fonseca-Madrigal *et al.*, 2014) and African catfish (*Clarias gariepinus*) (Oboh *et al.*, 2016).

The activity of Fads2 is highly dependent upon membrane fatty acid composition, since these are membrane-bound enzymes (Spector and Yorek, 1985; Vagner and Santigosa, 2011). Moreover, the choice of the active site of desaturases is also regulated through a competitive affinity hierarchy. The affinity of Δ5 and Δ6 Fads2 is higher for the n-3 than for the n-6 and n-9

series (Tocher, 2003; Zheng *et al.*, 2004a; Monroig *et al.*, 2010; Vagner and Santigosa, 2011; Hamid *et al.*, 2016). In addition, the affinity of the Δ6 Fads2 increases with the aliphatic chain length and the unsaturation degree (Glencross, 2009).

In addition to the Δ5 and Δ6 Fads2, two desaturases with Δ4 and Δ8 enzymatic activities have been reported. The Δ4 desaturase has the capacity to synthesise DHA directly from 22:5n-3 (Figure 1.1). The *fads2* gene encoding this protein has been isolated and characterised in several species, such as Senegalese sole (*Solea senegalensis*) and rabbitfish (Li *et al.*, 2010; Morais *et al.*, 2012; Fonseca-Madrigal *et al.*, 2014; Morais *et al.*, 2015; Castro *et al.*, 2016). The Δ8 desaturation acts in parallel to the classic n-6 and n-3 fatty acid bioconversions by desaturating 20:2n-6 and 20:3n-3 into 20:3n-6 and 20:4n-3, respectively, after the elongation of LA and ALA, respectively (Figure 1.1). A *fads2* gene encoding a desaturase with a Δ6 and Δ8 activities has been reported for teleost fish, with a higher Δ8 desaturation activity for marine species as compared to freshwater species (Monroig *et al.*, 2011).

#### *1.2.4.3.3 Elongases*

The processes of elongation and fatty acid neosynthesis are quite similar. However, the four steps of the elongation reaction are each catalysed by a single enzyme, whereas the fatty acid neosynthesis is performed with the Fas multienzyme complex. The process starts with the condensation of the precursor fatty acyl chain with malonyl-CoA and is followed by a reduction requiring NADPH, a dehydration, and a final second reduction (Jakobsson *et al.*, 2006). The first condensation being the rate-limiting step of the reaction, it is therefore considered as the actual "elongase" enzyme (Tocher, 2003; Jakobsson *et al.*, 2006; Castro *et al.*, 2016).

The "elongation of very-long chain" protein 2 (*elovl2*) and 5 (*elovl5*) genes, respectively, encode the fatty acid elongase 2 (Elovl2) and fatty acid elongase 5 (Elovl5) enzymes. These transmembrane enzymes are located in the endoplasmic reticulum and are involved in the n-6 and n-3 PUFA biosynthesis pathways (Jakobsson *et al.*, 2006), as presented in Figure 1.1. The Atlantic salmon *elovl5* cDNA reported by Hastings *et al.* (2004) encodes a 295-amino acid enzyme containing two transmembrane domains, a histidine box and the endoplasmic reticulum retention signal. These features are very similar to those of the mammalian ELOVL5. In rainbow

trout, the open reading frame of the *elovl5* cDNA reported by Gregory and James (2014) also encodes a 295-amino acid enzyme. Morais *et al.* (2009) characterised in Atlantic salmon an *elovl2* cDNA coding for a 287-amino acid enzyme, which presented high similarity with the mammalian ELOVL2 enzyme. A similar result was further obtained for rainbow trout (Gregory and James, 2014). Carmona-Antonanzas *et al.* (2011) reported the contribution of an fatty acid elongase 4 (*elovl4)* gene coding for the Elovl4 enzyme in Atlantic salmon. This enzyme is involved in the elongation of C20 and C22 fatty acids. However, its involvement is probably limited since *elovl4* is particularly expressed in eye and brain, but not in the liver.

In salmonids, Elovl2 catalyses the C20 and C22 fatty acid elongations and has a low activity towards C18 fatty acids. In contrast, Elovl5 catalyses the C18 and C20 fatty acid elongations and has a low activity towards C22 fatty acids (Morais *et al.*, 2009; Gregory and James, 2014). Besides the variation of affinity for the aliphatic chain length, Hastings *et al.* (2004) reported a preference of salmon Elovl5 for the n-3 fatty acid substrates whereas no difference was reported by Morais *et al.* (2009). In contrast, the rainbow trout Elovl5 and Elovl2 have a clear preference for the n-3 over the n-6 substrates (Gregory and James, 2014).

The *elovl2* and *elovl5* expression is predominant in the intestine, liver and brain of salmonids (Zheng *et al.*, 2005a; Morais *et al.*, 2009). Comparing both elongase genes, Gregory and James (2014) recorded a higher expression level of *elovl5* as compared to *elovl2* in the liver of adult rainbow trout.

### 1.2.5 Fatty acid storage and mobilisation

Rainbow trout, listed as an intermediate fish (between 2 and 10 % of lipids), accumulates and stores a large amount of lipids in its tissues, mainly in perivisceral adipose tissue and fillet (Médale, 2010). Lipids are mainly stored as TAG in adipocytes in the adipose tissue. In fillet, lipids can be stored as adipocytes within the myosepta (muscle connective tissue) or as intracellular lipid droplets in muscle cells (Jobling, 2007; Nanton *et al.*, 2007). In adult rainbow trout fed a diet with a fat content of around 25 %, the lipid content raises 8 % DM in fillet and 90 % DM in perivisceral adipose tissue (Barrado *et al.*, 2003). In wild rainbow trout, from 0.5 to 1.1 g of EPA and DHA is provided by 100 g of fillet. A similar content is reported for farmed fish fed a 100 % capelin oil feed (Bell and Tocher, 2009). As in

mammals, TAG are formed in the endoplasmic reticulum by the sequential esterification of two fatty acids to the glycerol-3-phosphate, which is catalysed by the glycerophosphate acyltransferase, followed by the cleavage of the phosphate group to form diacylglycerol and the esterification of a third fatty acid (Tocher, 2003).

When the energy requirements of fish exceed its dietary intake, such as during starvation or reproduction, the stored lipids are mobilised through the TAG degradation using the TAG lipase and the hormone-sensitive lipase (HSL), and the fatty acid β-oxidation to provide energy to cells (Tocher, 2003). The β-oxidation is an ATP-dependent process that mainly occurs in cell mitochondria and peroxisomes of liver and muscles, but also in the heart and gills (FrØyland *et al.*, 2000; Leaver *et al.*, 2008a) . The SFA are activated with CoA and transported into mitochondria through the carnitine palmitoyl-transferases I and II. The fatty acyl-CoA derivatives undergo a sequential cleavage of two-carbon units in four successive steps of dehydrogenation, hydration, dehydrogenation and cleavage by a thiolase to produce acetyl-CoA, $FADH_2$ and NADH. ATP is afterwards provided to the cells through the oxidation of acetyl-CoA via the citric acid cycle and the supply of $FADH_2$ and NADH to the respiratory chain. The β-oxidation of unsaturated fatty acids (MUFA and PUFA) follows the same four successive reactions but requires the additional intervention of an isomerase and a reductase. The β-oxidation of long chain (> 22 carbon atoms) fatty acids is initiated in peroxisomes and follows a similar four step process as for mitochondrial β-oxidation (Tocher, 2003; Lehninger *et al.*, 2008). The long chain fatty acids are thus catabolised to shorter-chain fatty acids before to be exported to mitochondria and completely oxidised. In contrast with mitochondria, the energy released in the first oxidative steps is not conserved as ATP, but is dissipated as heat (Leaver *et al.*, 2008a; Lehninger *et al.*, 2008).

In rainbow trout, β-oxidation appears to be a selective catabolism depending on the diet and the tissue (red or white muscles or liver) (Kiessling and Kiessling, 1993; Oxley *et al.*, 2005; Stubhaug *et al.*, 2007). According to Stubhaug *et al.* (2005), from 8 % to 25 % of the fatty acids taken up by the hepatocytes of Atlantic salmon are catabolised. SFA and MUFA are more readily β-oxidised than PUFA. LC-PUFA are less preferentially β-oxidised and among them, the n-3 family is even less β-oxidised than the n-6 family. In both families, the shorter chains are more quickly degraded than the

longer chains (Kiessling and Kiessling, 1993; Tocher, 2015). Accordingly, in Atlantic salmon hepatocytes isolated from fish fed a fish oil-based diet, a preferential use of radioactive 16:0, 18:1n-9, LA and AA for the mitochondrial β-oxidation has been observed as compared to the radioactive ALA, EPA and DHA (Stubhaug *et al.*, 2005). The DHA is considered as a poor substrate for β-oxidation due on one hand to the presence of the Δ4 double bond, which is resistant to the mitochondrial β-oxidation, and on another hand to its preferential storage in cell membranes (Madsen *et al.*, 1998). Moreover, the β-oxidation rate increases with the increased dietary concentration for most fatty acids (Stubhaug *et al.*, 2007).

## 1.3 Nutritional factors affecting fish metabolism and physiology

Feeding is a fundamental factor in salmonid farming. Indeed, unlike the semi-intensive cultured species partially feeding naturally preys in ponds, fish species in intensive farming, such as salmonids, rely on high protein and lipid containing feed to grow efficiently in aquaculture. The increased aquaculture production has led to an increased demand for fish feed, which is historically based on fish meal and fish oil. However, both of these ingredients have become expensive and difficult to source. Furthermore, given their status of finite marine resources, their use is widely criticised from a sustainability perspective (Tacon *et al.*, 2011; FAO, 2014a; Tocher, 2015). In terms of lipid supply, there is currently a need to optimise alternative feeding strategies in order to facilitate the production of farmed fish rich in n-3 LC-PUFA, whilst minimising the fish oil inclusion in fish feed. Some of these feeding strategies and their impact on fish lipid metabolism and physiology are described below, with a special focus on salmonids.

### 1.3.1 Fish oil use issue and alternative plant-derived oils

Historically, fish oil is one of the most important lipid sources in farmed fish feed (Lovell, 2003). This is explained by the n-3 LC-PUFA-rich fatty acid profile of fish oil, which enables to perfectly match with the fish fatty acid requirements and induces the production of farmed fish rich in health promoting n-3 LC-PUFA for the human consumer (NRC, 2011). In 2012, about 75 % of the fish oil global production was intended to aquaculture, while 22 % were used for direct human consumption (FAO, 2014a; Tocher, 2015), as observed in Figure 1.2. Even though the salmonid culture represents only a small part of the global aquaculture production, salmonid farming consumes about 60 % of the total fish oil used in commercial fish feed (Tacon *et al.*, 2011; Tocher, 2015) (Figure 1.2).

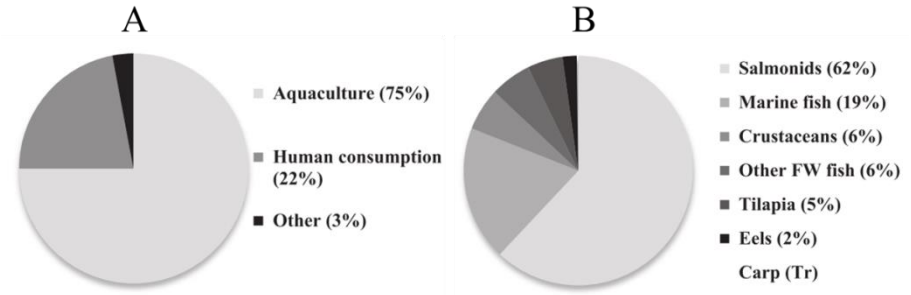


**Figure 1.2.** Global use of fish oil in 2012. A. Major sectors consuming fish oil in 2012; B. Consumption of fish oil within the aquaculture sector in 2012. FW, freshwater; Tr, trace. Adapted from Tocher (2015).

The fish oil production is based on a few, fast growing, short lived, productive stocks of small pelagic fish, such as Peruvian anchovy, capelin, sandeel and sardines, in the subtropical and temperate regions (De Silva and Soto, 2009). In addition, about one-third of the current fish meal and oil productions are derived from fish remains or other fish by-products, such as heads, tails and bones, and not from whole fish (FAO, 2014a).

The primary constraint with the fish oil use is that it is a finite and limited marine resource and therefore has become rare and expensive, economically and environmentally speaking (Turchini *et al.*, 2009). As observed in Figure 1.3, the production of fish oil has declined since 2011, due to increased regulation and reduced quotas, as well as environmental influences, and reached 850 000 tonnes in 2015 (FAO, 2012; IFFO, 2016; Shepherd *et al.*, 2016). With the high demand for fish oil, its price has steadily increased since 2009, as observed in Figure 1.4, and amounted about USD 2000 per tonne in 2015 (Hixson, 2014; Marine Harvest, 2016).

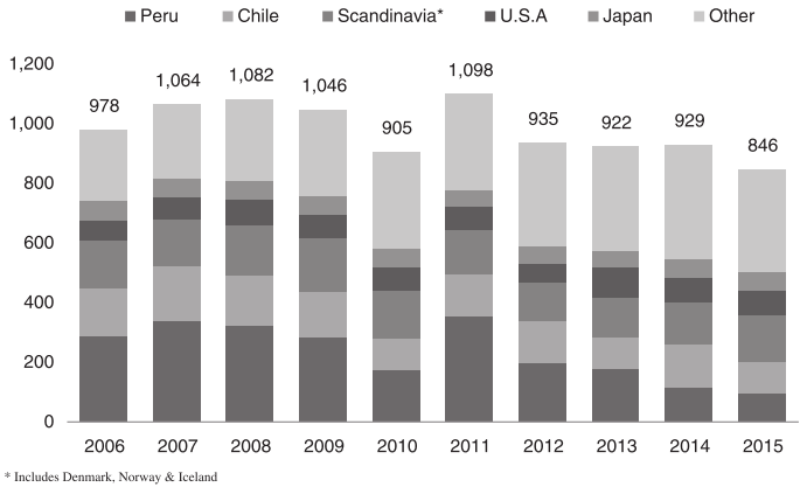


**Figure 1.3.** Annual and global fish oil production by major producers from 2005 to 2013 (tonnes × 1000) (Shepherd *et al.*, 2016).

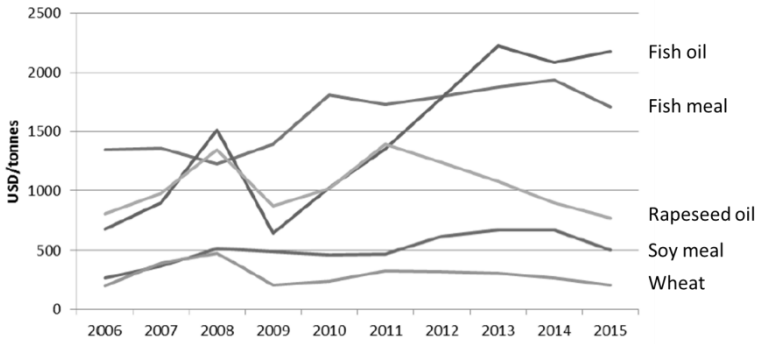


**Figure 1.4.** Prices of fish oil, fishmeal, rapeseed oil, soy meal and wheat delivered in Europe, from 2006 to 2013. Adapted from Marine Harvest (2016).

The continued expansion of the aquaculture sector forces the fish farmers to rely to a lower extent on the expensive and limited availability of fish oil as dietary lipid source in aquaculture feed and searching for alternative lipid sources is thus required. The incorporation of alternative lipid sources into commercial fish feed is now a common practice for many cultured fish species. As observed in Figure 1.5, the Norwegian salmon feed was entirely based on fish oil as lipid source in the 1990s and 2000s, whereas, from 2010 on, the inclusion of fish oil progressively decreased to achieve only about 40 % of the dietary lipid sources in 2013 (Ytrestøyl *et al.*, 2015). Therefore, the fish oil inclusion in farmed fish feed shows a clear downward trend since 2011 despite the expansion of the aquaculture sector (Tacon and Metian, 2008; FAO, 2014a; Shepherd *et al.*, 2016), as observed in Figure 1.6.

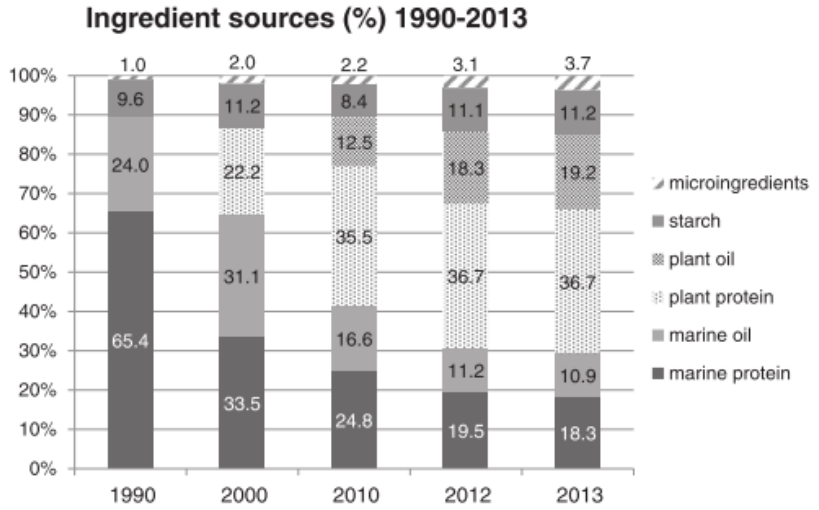


**Figure 1.5.** Dietary sources in Norwegian salmon feed from 1990 to 2013. Each ingredient type is shown as its percentage of the total diet (Ytrestøyl *et al.*, 2015).

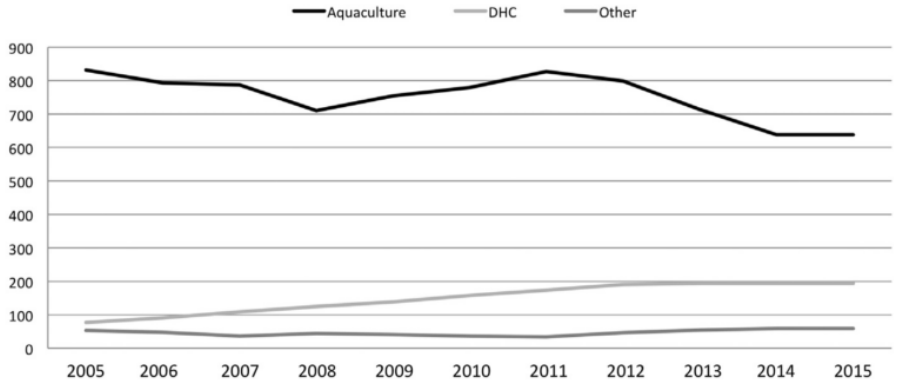


**Figure 1.6.** World fish oil consumption (tonnes × 1000) by aquaculture, direct human consumption (DHC) and other uses from 2005 to 2015 (IFFO, 2016; Shepherd *et al.*, 2016).

The challenges in fish oil replacement are, on one hand, to provide sufficient amounts of dietary EFA for the optimal growth and development of fish and, on the other hand, to still supply n-3 LC-PUFA-rich fish to the human consumer. One of the key sustainable alternatives to fish oil comes from terrestrial plant-derived oils, predominantly crop-derived oils (Gatlin Iii *et al.*, 2007; Turchini *et al.*, 2009; Hixson, 2014). The major constraint with fish oil replacement by plant-derived oils is their difference in terms of n-3 PUFA composition. Indeed, the fatty acid profile of plant-derived oils is not equivalent to that of fish oil since plant-derived oils are devoid of n-3 LC-

PUFA. Therefore, the research on the effective replacement of fish oil by plant-derived oils in fish feed has been extensive (Hixson, 2014). The most frequently tested plant-derived oils in fish feed as alternatives to fish oil are linseed oil (the richest source of ALA), olive oil (the richest source of OLA), palm oil (the richest source of 16:0 and also rich in OLA), rapeseed oil (rich in OLA), canola oil (low 22:1n-9 rapeseed oil), soybean oil (rich in LA), sunflower oil (rich in LA) (Glencross, 2009; Hixson, 2014). Other promising plant-derived oils are oils from *Camelina sativa* (rich in ALA) (Hixson *et al.*, 2014; Betancor *et al.*, 2015), *Perilla frutescens* (60 % ALA) (Shin and Kim, 1994), echium (32 % ALA and 14 % 18:4n-3) (Glencross, 2009), and also from genetically modified plant-derived oils with a higher n-3 LC-PUFA content (Hixson, 2014), such as the genetically modified *Camelina sativa* oil rich in EPA and DHA (Ruiz-Lopez *et al.*, 2014). The detailed fatty acid composition of some plant-derived oils is presented in Table 1.3.

**Table 1.3.** Typical fatty acid composition (in % of total fatty acids) of some plant-derived oils used in fish feed formulations

| Fatty acid | Soybean | Palm | Rapeseed | Sunflower | Linseed | Olive | Echium |
|---|---|---|---|---|---|---|---|
| 16:0 | 10.3 | 43.5 | 3.1 | 5.9 | 5.3 | 11 | 7 |
| 18:0 | 3.8 | 4.3 | 1.5 | 4.5 | 4.1 | 2.2 | 4 |
| 16:1n-7 | 0.2 | 0.3 | 0 | 0 | 0 | 0.8 | 0 |
| 18:1n-9 + 18:1n-7 | 22.8 | 36.6 | 60 | 19.5 | 20.2 | 72.5 | 16 |
| 20:1n-9 + 20:1n-11 | 0.2 | 0.1 | 1.3 | 0 | 0 | 0 | 1 |
| 22:1n-9 + 22:1n-11 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| 18:2n-6 | 51 | 9.1 | 20.2 | 65.7 | 12.7 | 7.9 | 15 |
| 20:4n-6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18:3n-3 | 6.8 | 0.2 | 12 | 0 | 53.3 | 0.6 | 32 |
| 18:4n-3 | 0 | 0 | 0 | 0 | 0 | 0 | 14 |
| 20:5n-3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22:6n-3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| SFA | 15.1 | 51.6 | 7.4 | 10.6 | 9.4 | 14.1 | 11 |
| MUFA | 23.2 | 37 | 62.3 | 19.5 | 20.2 | 73.3 | 16 |
| PUFA | 61.7 | 11.4 | 32.2 | 69.9 | 70.4 | 12.6 | 73 |
| LC-PUFA | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| n-3 PUFA | 6.8 | 0.2 | 12 | 0 | 53.3 | 0.6 | 32 |
| n-6 PUFA | 51 | 9.1 | 20.2 | 65.7 | 12.7 | 7.9 | 15 |

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids; n-3 PUFA, polyunsaturated fatty acids of the n-3 family; n-6 PUFA, polyunsaturated fatty acids of the n-6 family. Adapted from NRC (2011).

Besides the difference in fatty acid composition, the replacement of fish oil by plant-derived oils induces also the absence of some compounds in fish diets, such as vitamin A, vitamin D and cholesterol (Médale *et al.*, 2013). Diets lacking such compounds could also impact on fish lipid metabolism (Norambuena *et al.*, 2013). On a human health aspect, the fish oil replacement by plant-derived oils induces a significant reduction of organic and inorganic contaminants in fish, which bioaccumulate many of these contaminants (Nøstbakken *et al.*, 2015). Considering Atlantic salmon farmed in 2014, the concentrations in polychlorinated biphenyls (PCBs) and mercury were reduced by about 30 and 50 %, respectively, as compared to

the corresponding levels reported in 2006, with the replacement of marine ingredients by plant-based ingredients (Shepherd *et al.*, 2016).

Aside from plant-derived oils, other fish oil alternatives include livestock tallow or fat, zooplankton oils and algal oils (Glencross, 2009; Turchini *et al.*, 2009; Miller *et al.*, 2010).

Unlike fish oil, the production of plant-derived oils has considerably increased the last few years (Turchini *et al.*, 2009). The largest production of plant-derived oils comprises palm oil (world production of 50 million tons in 2010), soybean oil (40 million tons), rapeseed oil (19 million tons) and sunflower oil (10 million tons) (Glencross, 2009; Médale *et al.*, 2013). With the exception of sunflower oil, the price of the three other major oils is generally lower than the price of fish oil (Turchini *et al.*, 2009). As observed in Figure 1.4, in 2015, the average price of rapeseed oil was about USD 800 per tonne (Marine Harvest, 2016). Considering salmonid farming, plant-derived oils are currently included to the fish feed at about 19 % of the total diet formulation, which represents about 60 % of the total lipid inclusion (Figure 1.5). Rapeseed oil is currently the most plant-derived oil used as alternative to fish oil. Palm oil is also used, but to a lesser extent (Marine Harvest, 2016; Shepherd *et al.*, 2016).

Although considered as the best alternative to fish oil, the inclusion of plant-derived oils in fish feed still results in fish with a reduced content in n-3 LC-PUFA, particularly EPA and DHA, which is detrimental in terms of fish health and fish benefits for humans. The impacts of plant-derived oils as lipid source in fish feed on the fish metabolism and physiology is reported in the following section, with a special focus on salmonids.

### 1.3.2 Dietary lipid source impact on fish lipid metabolism and physiology

#### 1.3.2.1 Fish lipid metabolism and physiology

*Feed acceptance*

The feed acceptance is an important factor in fish nutrition as it affects the fish growth performance through the feed intake. Feed acceptance is based on nutritional, chemical and physical characteristics (Jobling *et al.*, 2007). Fish can distinguish diets and select the one which corresponds the best to their requirements. In a two-choice self-feeding trial, rainbow trouts were

able to discriminate between a fish oil diet and plant-derived oil diets, with a preference for the fish oil diet over the rapeseed oil diet and then the linseed oil diet (Geurden *et al.*, 2005). Moreover, in a no-choice feeding over a 80-day period using self-feeders, the comparison of the number of feed demands over the trial showed more than 30 % lower activity for fish fed a linseed oil diet than in groups fed fish oil, rapeseed oil or olive oil diets. However, this difference in feeding behaviour was not related to difference in feed intake and fish growth, which were similar between all groups (Geurden *et al.*, 2007).

*Digestion and absorption*

Feeding fish with a plant-derived oil diet has a clear effect on the membrane fatty acid composition of enterocytes and consequently on the fatty acid uptake by enterocytes. Geurden *et al.* (2009) reported an increased transepithelial passage rate of ALA in the intestine of fish fed a linseed oil diet, as compared to rapeseed and fish oil diets. Moreover, a higher uptake than export rate was observed in enterocytes of rainbow trout with the presence of lipid droplet accumulations when fish fed plant-derived diets (Caballero *et al.*, 2002). The accumulation of lipid droplets in enterocytes from pyloric caeca and midgut has also been observed in Arctic char fed a linseed oil diet (Olsen *et al.*, 1999) and gilthead seabream (*Sparus aurata*) fed a plant-derived oil diet (Caballero *et al.*, 2003). The lipid accumulation in enterocytes may be explained by an absorption rate higher than the lipoprotein synthesis rate (Olsen *et al.*, 1999).

The effects that the inclusion of plant-derived oils in fish diet has on intestinal lipid absorption directly impact the apparent digestibility of fatty acids. Divergent effects on fatty acid digestibility are reported depending on the nature of the dietary lipid source. An increased apparent digestibility of fatty acids was reported in rainbow trout (Geurden *et al.*, 2009) and Atlantic salmon (Menoyo *et al.*, 2007) fed a linseed oil diet, in comparison with fish fed a fish oil diet. Conversely, Atlantic salmon fed sunflower oil or palm oil-based diets had lower apparent digestibility values for a large number of fatty acids (*i.e.* 16:0, OLA, ALA, EPA and DHA), as compared to fish fed a capelin oil diet (Torstensen *et al.*, 2000). In this study, the apparent digestibility of SFA decreased from 86 % when fish fed capelin oil diet to 74 % and 18 % when supplied in a sunflower or palm oil diet, respectively (Torstensen *et al.*, 2000). In rainbow trout, the replacement of 60 % of dietary fish oil by rapeseed oil induced higher apparent digestibility of SFA,

MUFA, LA, ALA, whereas EPA was not impacted, and DHA had a lower apparent digestibility (Karalazos *et al.*, 2011).

*Lipid transport*

The fatty acid composition of lipoproteins and their plasma level are highly influenced by the dietary fatty acid composition (Torstensen *et al.*, 2000; 2004a; Vegusdal *et al.*, 2005; Richard *et al.*, 2006; Jordal *et al.*, 2007). Studies previously reported that the fatty acid composition of VLDL and LDL was more affected by a dietary fatty acid composition change than it was on HDL (Torstensen *et al.*, 2000; 2004a). In rainbow trout, a decrease in plasma LDL level was observed in fish fed a plant-based diet formulated with rapeseed, palm and linseed oils (55:30:15). Moreover, the expression of the LDL receptor gene in the liver was reduced (Richard *et al.*, 2006). In Atlantic salmon fed a plant-derived oil diet formulated with rapeseed, palm and linseed oils (55:30:15), the plasma LDL and VLDL levels were also reduced, as compared to fish fed a fish oil diet (Jordal *et al.*, 2007). This may be explained by a reduced hepatic synthesis of lipoproteins and secretion of TAG from hepatocytes, or by an increased uptake of lipoproteins by peripheral tissues (Jordal *et al.*, 2007). Vegusdal *et al.* (2005) reported that the rate of secretion of TAG was increased in hepatocytes isolated from Atlantic salmon fed a soybean oil diet than from fish fed a fish oil diet. Moreover, the authors showed that a high OLA level in the culture medium increased both hepatic TAG synthesis and excretion from hepatocytes, whereas a high EPA level reduced the hepatic TAG secretion (Vegusdal *et al.*, 2005). In terms of fatty acid uptake, hepatocytes from Atlantic salmon were less efficient when fish from which cells were isolated had been fed a plant-derived diet, as compared to a fish oil diet (Stubhaug *et al.*, 2005).

*Fatty acid mobilisation*

The dietary replacement of fish oil by plant-derived oils may affect the β-oxidation capacity in fish tissues (Turchini *et al.*, 2009). An increased β-oxidation of OLA, LA, ALA and EPA was reported in hepatocytes of Atlantic salmon fed a 75 % plant-derived oil diet formulated with rapeseed, palm, and linseed oils (3.7:2:1), as compared to a fish oil diet (Stubhaug *et al.*, 2005). In contrast, the expression of genes related to β-oxidation was reduced in white muscle of Atlantic salmon fed a diet formulated with rapeseed, palm and linseed oils (55:30:15) (Torstensen *et al.*, 2009). Conversely, in red muscle of Atlantic salmon, no difference of mitochondrial

β-oxidation was reported between fish fed diets formulated with capelin, sunflower or palm oils (Torstensen *et al.*, 2000). Similarly, no effect of the dietary lipid source was reported on the β-oxidation in isolated hepatocytes and enterocytes of Atlantic salmon fed a fish oil or a plant-derived oil blend diet (Tocher *et al.*, 2003).

*Neosynthesis*

The neosynthesis is modulated by the dietary fatty acid content and composition through effects on Fas and acetyl-CoA carboxylase activities (Alvarez *et al.*, 2000; Rollin *et al.*, 2003a; Jordal *et al.*, 2007). In rainbow trout hepatocytes, the addition of 50 µM ALA to the culture medium inhibited the enzymatic activity of Fas by a factor of 3.5 with regard to the basal activity. Similarly, the addition of EPA and DHA (50 µM) to the culture medium induced the inhibition of Fas activity by a factor of 35 and 5.4, respectively (Alvarez *et al.*, 2000). In coho salmon (*Oncorhynchus kisutch*) liver, the enzymatic activity of Fas was depressed as the dietary lipid content increased (Lin *et al.*, 1977). With Atlantic salmon, an *in vivo* study reported that the activity of Fas was reduced in fish fed a plant-derived diet formulated with rapeseed, palm and linseed oils (55:30:15) for 16 months, as compared to fish fed a fish oil diet (Jordal *et al.*, 2007). Conversely to the above studies, no effect on Fas activity was reported in the liver of rainbow trout fed diets formulated with fish oil or a blend of plant-derived oils (Richard *et al.*, 2006).

Similarly to the effects on Fas activity, the acetyl-CoA carboxylase was inhibited by a diet with a high lipid content (Rollin *et al.*, 2003a). This was observed in the liver and intestine but not in other tissues (adipose tissue, kidney, red fillet, dorsal and ventral white fillets).

*Gene expression and enzymatic activity of desaturases and elongases*

The Δ6 and Δ5 Fads2 and elongases, namely Elovl2, Elovl4 and Elovl5, are subject to nutritional regulation. This regulation has been extensively studied to test whether these enzyme activities could compensate for the reduced n-3 LC-PUFA content in fish fed plant-derived oil diets (Vagner and Santigosa, 2011; Tocher, 2015). The substitution of fish oil by plant-derived oils, including linseed oil (Tocher *et al.*, 2002; Rollin *et al.*, 2003b; Tocher *et al.*, 2003; Zheng *et al.*, 2004b), rapeseed oil (Bell *et al.*, 2001; Tocher *et al.*, 2003; Zheng *et al.*, 2005a; 2005b), sunflower oil (Tocher *et al.*, 1997),

soybean oil (Leaver *et al.*, 2008b), olive oil (Buzzi *et al.*, 1996; Tocher *et al.*, 1997), palm oil (Tocher *et al.*, 2004), or sesame oil (Köse and Yildiz, 2013), as sole source or as a blend, in diets of salmonids has been shown to result in increased activity along the n-6 and n-3 desaturation and elongation pathways. In rainbow trout hepatocytes, an increased Δ6 Fads2 activity was observed in hepatocytes from fish fed an olive oil diet, as compared to a fish oil diet (Buzzi *et al.*, 1996). In the same species, increased desaturation and elongation activities were reported in hepatocytes of fish fed diets with increased replacement of fish oil by palm oil (Tocher *et al.*, 2004). In Atlantic salmon hepatocytes, the Δ5 Fads2 activity, as well as the Elovl5 activity, increased in a graded manner in response to increased dietary linseed oil inclusion (Zheng *et al.*, 2004b). In rainbow trout, the apparent *in vivo* enzymatic activities of Δ5 and Δ6 Fads2 were increased with the replacement of fish oil by canola oil (low 22:1n-9 rapeseed oil) up to 90 % in the diet (Turchini *et al.*, 2013). In the same species, increased apparent *in vivo* enzymatic activities of Δ6 and Δ5 Fads2 were reported in fish fed a plant-based diet formulated with linseed and sunflower oils (58:42), in comparison with fish fed a fish oil diet (Thanuthong *et al.*, 2011a).

Similarly to the impact of the dietary lipid source on desaturase and elongase enzymatic activities, the gene expression of *fads2*, *elovl2* and *elovl5* is also increased in tissues of fish fed plant-derived oil diets (Zheng *et al.*, 2005b; Leaver *et al.*, 2008b; Morais *et al.*, 2009; Monroig *et al.*, 2010; Gregory *et al.*, 2016). The *fads2* gene expression was shown to be increased in liver, fillet, intestine and adipose tissue of Atlantic salmon fed a diet in which 75 % of fish oil was replaced by a blend of rapeseed, palm and linseed oil (3.7:2:1), as compared to fish fed a fish oil diet (Zheng *et al.*, 2005a). Conversely, the elongase gene expression was reduced in most tissues, mainly heart, gill and brain. In rainbow trout, the liver transcript level of *fads2* coding for a Δ6 Fads2 was higher in trout fed a linseed oil diet, as compared to trout fed a fish oil diet (Seiliez *et al.*, 2001). Similarly, the expression of *fads2* coding for a Δ5 Fads2 was higher in the liver of fish fed a diet in which fish oil was replaced by 75 % of rapeseed oil than in fish fed a 100 % fish oil diet (Jordal *et al.*, 2005). The up-regulation in fish fed plant-derived oil diets is due mainly to relieving the suppression of gene expression induced by dietary n-3 LC-PUFA (Seiliez *et al.*, 2001; Vagner and Santigosa, 2011; Tocher, 2015). In Atlantic salmon, the hepatic expression of *fads2* coding for a Δ5 Fads2 and of *elovl5* was positively correlated with dietary ALA in fish fed a linseed oil diet and negatively

correlated with dietary n-3 LC-PUFA in fish fed a fish oil diet (Zheng *et al.*, 2004b).

Considering the dietary cholesterol effects, the exogenously added cholesterol in a plant-derived oil diet induced a higher transcription rate of *fads2* in the liver of rainbow trout and an increased apparent *in vivo* elongase activity (Norambuena *et al.*, 2013). In Atlantic salmon hepatocytes supplemented with radioactive ALA, a higher conversion of ALA into n-3 LC-PUFA was observed when fish were previously fed a 100 % plant-derived oil diet as compared to those fed a 65 % plant-derived oil diet (35 % of fish oil) (Sanden *et al.*, 2016). However, the EPA and DHA contents in liver phospholipids were lower for fish fed a 100 % plant-derived diet than 65 %. The authors concluded that the reduced cholesterol content induced by the increased dietary plant-derived oil inclusion resulted in metabolic perturbations in the liver of Atlantic salmon (Sanden *et al.*, 2016).

The dietary fatty acids can affect the desaturase and elongase gene expressions and activities through numerous direct and indirect mechanisms including changes in membrane lipid composition, eicosanoid production, oxidant stress, nuclear receptor activation or covalent modification of specific transcription factors (Vagner and Santigosa, 2011). It is still unclear whether it is the desaturation product reduction or the increased substrate supply that drives the nutritional regulation of desaturases and elongases (Vagner and Santigosa, 2011). However, in a recent study on rainbow trout, Gregory *et al.* (2016) reported that the gene expressions of *fads2* coding for a Δ6 Fads2, *elovl5* and *elovl2* were higher in fish fed diets with a high ALA content and no dietary EPA and DHA, as compared to a diet containing ALA, EPA and DHA. Moreover, the expressions of *fads2* coding for Δ5 or Δ6 Fads2, *elovl5* and *elovl2* were down-regulated by dietary EPA and DHA, with a more pronounced effect from DHA, as compared to a low n-3 PUFA diet (Gregory *et al.*, 2016).

*Transcription factors*

The fatty acid metabolism is regulated by different transcription factors, such as the sterol regulatory element binding proteins (Srebp), the liver X receptor (Lxr), the peroxisome proliferator-activated receptors (Ppar) and the hepatocyte nuclear factor 4α (Hnf4α) (Nakamura and Nara, 2004; Leaver *et al.*, 2008a; Cruz-Garcia *et al.*, 2011; Carmona-Antoñanzas *et al.*, 2014; Dong *et al.*, 2016). A transcription factor is a protein, which can bind to

regulatory regions of DNA, known as response elements, near the target gene and consequently regulates the transcription of the target gene into mRNA (Lehninger *et al.*, 2008).

Lxr are ligand-activated transcription factors of the nuclear receptor family. A single Lxr subtype has been identified in various fish species (Reschly *et al.*, 2008) and oxysterols have been reported as natural Lxr ligands (Carmona-Antoñanzas *et al.*, 2014). The complex ligand-Lxr forms an heterodimer with the retinoid X receptor before binding to the liver X receptor response element of the target genes in order to modulate the gene transcription (Carmona-Antoñanzas *et al.*, 2014). The Lxr activation induces the up-regulation of the lipid neosynthesis through increased *fas* expression, of the LC-PUFA biosynthesis through increased expression of *fads2* coding for Δ5 and Δ6 Fads2 and of the cholesterol metabolism (Cruz-Garcia *et al.*, 2011; Carmona-Antoñanzas *et al.*, 2014). It has been reported that LA and OLA down-regulated the *lxr* expression in rainbow trout fillet (Cruz-Garcia *et al.*, 2011) whereas an up-regulation of *lxr* expression was observed in adipose tissue (Cruz-Garcia *et al.*, 2012).

Srebp are membrane-bound transcription factors regulating the gene expression of enzymes involved in the fatty acid and cholesterol biosynthesis. Two different types of *srebp* genes have been identified: *srebp1* and *srebp2*, as observed in Atlantic salmon (Minghetti *et al.*, 2011). Srebp1c up-regulates the genes required for *de novo* lipogenesis and genes encoding Δ5 and Δ6 Fads2 and Elovl5 whereas Srebp2 is involved in the regulation of cholesterol biosynthesis (Amemiya-Kudo *et al.*, 2002; Zheng *et al.*, 2009; Minghetti *et al.*, 2011; Carmona-Antoñanzas *et al.*, 2014; Zhang *et al.*, 2016a). Srebp isoforms are present as inactivated proteins on the nuclear envelope or the endoplasmic reticulum membrane. A proteolytic cleavage is required to release the mature form of Srebp for translocation to the nucleus and further gene regulation (Brown and Goldstein, 1997). Srebp proteins induce the transcription of the target gene by binding to the sterol regulatory element, located in the response element of the target gene (Matsuzaka *et al.*, 2002; Nakamura and Nara, 2004; Zheng *et al.*, 2009). The *srebp1* and *srebp2* expression is increased by Lxr, as observed in the SHK-1 cell line derived from Atlantic salmon head kidney (Minghetti *et al.*, 2011; Carmona-Antoñanzas *et al.*, 2014). In this cell line, it has been reported that this transcription factor association, called "Lxr-Srebp1 pathway", increased the expression of *fads2* encoding for Δ5 and Δ6 Fads2 and had no impact on

*elovl5* expression (Carmona-Antoñanzas *et al.*, 2014). PUFA are presumed to inhibit the activation of Srebp1 and 2 by different mechanisms and LC-PUFA are more potent inhibitors than the precursor fatty acids (Nakamura and Nara, 2004; Geay *et al.*, 2010; Minghetti *et al.*, 2011; Carmona-Antoñanzas *et al.*, 2014). Srebp have therefore a mediator role in the down-regulation of *fads2* expression induced by LC-PUFA (Nakamura and Nara, 2004).

Ppar are ligand-dependent transcription factors of the nuclear receptor superfamily and possess 3 subtypes, Ppar alpha (Pparα), beta (Pparβ) and gamma (Pparγ) (Andersen *et al.*, 2000; Berger and Moller, 2002; Batista-Pinto *et al.*, 2005; Leaver *et al.*, 2007). The Pparα isoform is abundantly present in hepatocytes and plays a critical role as activator in the cellular uptake of fatty acids, the mitochondrial and peroxisomal β-oxidations and the fatty acid biosynthesis (Jeng and Hou, 2005; Leaver *et al.*, 2008a; Vagner and Santigosa, 2011). The most effective Pparα classical ligands are the eicosanoids and the C16 to C20 fatty acids with several double bonds (Berger and Moller, 2002; Kennedy *et al.*, 2006; Leaver *et al.*, 2008a; Vagner and Santigosa, 2011). In rainbow trout, the incubation of liver slices with fatty acids showed that the expression of *pparα* was up-regulated by SFA, MUFA, ALA, AA and DHA, and down-regulated by LA and EPA (Coccia *et al.*, 2014). The complex ligand-Pparα forms an heterodimer with the retinoid X receptor in the nucleus before binding to the peroxisome proliferator response element of the target genes in order to modulate the gene transcription (Berger and Moller, 2002; Vagner and Santigosa, 2011). Pparα has a mediator role in the up-regulation of the *fads2* gene expression induced by PUFA (Matsuzaka *et al.*, 2002; Kennedy *et al.*, 2006; Vagner *et al.*, 2009; Vagner and Santigosa, 2011). In brown trout, Batista-Pinto *et al.* (2005) identified the expression of *pparα* in liver, muscle and heart, *pparβ* in liver, muscle, heart, testis and kidney and *pparγ* in liver and kidney.

Dietary lipids may affect desaturase and elongase gene expressions through different ways. The fatty acids can bind to Pparα as ligands, be converted into eicosanoids, which are other Pparα ligands and affect Srebp1c. The expression of *fads2* is under Srebp1c and Pparα regulations (Matsuzaka *et al.*, 2002). This *fads2* regulation is paradoxical because both Srebp1c and Pparα induce normally exclusive sets of genes (Nakamura and Nara, 2004). Moreover, PUFA, including LC-PUFA, induce opposite effects on the regulation of *fads2* expression since PUFA are Pparα ligands and therefore

up-regulate *fads2* expression, whereas they inhibit the Srebp1c action and therefore down-regulate the *fads2* expression (Nakamura and Nara, 2004; Kennedy *et al.*, 2006).

Recently, Dong *et al.* (2016) were the first to demonstrate the presence of Hnf4α, a transcription factor belonging to the nuclear receptor hormone superfamily (Leaver *et al.*, 2008a), as transcription factor inducing the expression of a *fads2* gene with a Δ4 desaturation activity in rabbitfish.

The fish fatty acid metabolism can also be regulated through post-transcriptional regulation of gene expression by microRNAs (Zhang *et al.*, 2014; Zhang *et al.*, 2016b). These are short non-coding RNA molecules with about 22 nucleotides, which can bind to target protein-coding mRNA through their "seed region" and consequently inhibit the expression of the target gene via translation repression and/or mRNA decay (Ambros, 2004; Zhang *et al.*, 2014). About 200 microRNAs have been identified and characterised in Atlantic salmon (Andreassen *et al.*, 2013). The first study evaluating the effect of microRNAs on LC-PUFA biosynthesis in teleost fish has been conducted by Zhang *et al.* (2014). These authors demonstrated that mir-17 targeted and down-regulated the gene encoding Δ4 desaturation activity in rabbitfish liver. The expression of mir-17 was increased when primary cells were incubated with ALA whereas a reduced mir-17 expression was observed with LA, EPA and DHA (Zhang *et al.*, 2014). More recently, it has been reported that the mir-33 microRNA up-regulated the expression of *fads2* in rabbitfish hepatocytes and that the mir-33 was expressed in parallel to the *srebp1* expression (Zhang *et al.*, 2016b). The authors suggested that miR-33 acted on *fads2* expression by repressing negative regulators of *fads2* expression. An increased mir-33 expression was reported in hepatocytes incubated with ALA, as compared to EPA and DHA (Zhang *et al.*, 2016b).

*Growth*

Several studies on salmonids have reported that the substitution of fish oil by plant-derived oils in the diet has no impact on fish growth performance if adequate amounts of EFA are supplied through 20 % of fish meal in the diet (Richard *et al.*, 2006; Bell and Tocher, 2009; Corraze and Kaushik, 2009; Turchini *et al.*, 2009; Médale *et al.*, 2013). In rainbow trout, the replacement of fish oil by linseed oil (Richard *et al.*, 2006; Geurden *et al.*, 2009; Thanuthong *et al.*, 2011b; Francis *et al.*, 2014; Yildiz *et al.*, 2014; Gregory *et*

*al.*, 2016), sunflower oil (Thanuthong *et al.*, 2011b; Francis *et al.*, 2014; Yildiz *et al.*, 2014), rapeseed oil (Caballero *et al.*, 2002; Richard *et al.*, 2006), olive oil (Francis *et al.*, 2014), canola oil (Turchini *et al.*, 2013), palm oil (Caballero *et al.*, 2002; Richard *et al.*, 2006; Francis *et al.*, 2014), and sesame oil (Köse and Yildiz, 2013; Yildiz *et al.*, 2014), as sole dietary lipid source or as a blend had no impact on fish growth performance. Similarly, in Atlantic salmon, the substitution of fish oil by linseed oil (Rollin *et al.*, 2003b; Bell *et al.*, 2004; Menoyo *et al.*, 2005; 2007; Leaver *et al.*, 2008b), sunflower oil (Rollin *et al.*, 2003b), soybean oil (Torstensen *et al.*, 2000; Leaver *et al.*, 2008b), palm oil (Torstensen *et al.*, 2000), rapeseed oil (Bell *et al.*, 2001; Torstensen *et al.*, 2004a; Leaver *et al.*, 2008b), or oil from transgenic *Camelina sativa*, rich in ALA and EPA (Betancor *et al.*, 2015), had no negative effect on fish growth performance.

In most reported studies, the replacement of fish oil by plant-derived oils in fish feed slightly affected the feed intake of fish (Turchini *et al.*, 2009). No effect on the feed intake was reported in rainbow trout when dietary fish oil was replaced by a blend of plant-derived oils (linseed and sunflower oils, 58:42) (Thanuthong *et al.*, 2011b) or by linseed, olive, sunflower or palm oil as sole dietary lipid source (Francis *et al.*, 2014). Moreover, no effects on fish body weight gain, specific growth rate (SGR) and feed efficiency (FE) were reported for fish fed a diet formulated with linseed and sunflower oils (58:42), in comparison with fish fed a fish oil diet (Thanuthong *et al.*, 2011b). In Atlantic salmon, the replacement of fish oil by linseed, olive or sunflower oil in the diet had no effect on the fish body weight gain or the daily growth coefficient (DGC) (Rollin *et al.*, 2003b). The hepato-somatic index (HSI) was not affected when Atlantic salmon were fed a linseed oil diet (Menoyo *et al.*, 2005) or in rainbow trout fed plant-derived oil diets (Caballero *et al.*, 2002; Thanuthong *et al.*, 2011b). Similarly, diets formulated with linseed, sunflower, sesame, rapeseed or soybean oil had no impact on HSI in rainbow trout (Francis *et al.*, 2014; Yildiz *et al.*, 2014) and Atlantic salmon (Leaver *et al.*, 2008b).

In contrast with the above mentioned studies and observations, the concomitant replacement of dietary fish meal and fish oil is known to reduce the fish growth performance, which could be linked to a combination of lower feed intake and FE (Panserat *et al.*, 2009). The replacement of fish meal has indeed been reported to have a significant impact on rainbow trout growth, as compared to the fish oil substitution (Panserat *et al.*, 2008).

*Fish and tissue lipid contents*

Generally, the increase in lipid content of salmonid diets leads to an increased lipid deposition, mainly as TAG, in perivisceral adipose tissue and fillet whereas the lipid content of liver is impacted to a lesser extent (Corraze and Kaushik, 2009). In contrast, the substitution of fish oil by plant-derived oils in salmonid diets induces a limited impact on the lipid content of fish and its tissues (Richard *et al.*, 2006; Corraze and Kaushik, 2009; Francis *et al.*, 2014). However, the effects of fish oil substitution appear contradictory in the literature. For instance, the lipid content of whole body Atlantic salmon was not affected by the dietary lipid source (linseed, sunflower or olive oil in the diet) (Rollin *et al.*, 2003b). Similarly, the lipid content of liver of rainbow trout was not impacted by the gradual replacement of fish oil by palm oil (Tocher *et al.*, 2004) or in the fillet by the gradual replacement of fish oil by a blend plant-derived oil diet (Thanuthong *et al.*, 2011b). In contrast, in rainbow trout fed a 100 % plant-based diet, the lipid content in the liver, mainly composed of PL, was reduced whereas it increased in the adipose tissue and carcass, mainly composed of TAG (Lazzarotto *et al.*, 2015). Similar observations were reported for rainbow trout fed a diet formulated with fish meal and fish or plant-derived oils (Köse and Yildiz, 2013). In the fillet of the same species, a reduced lipid content was reported with the replacement of dietary fish oil by sunflower, sesame or linseed oil (Yildiz *et al.*, 2014). In Atlantic salmon, the replacement of fish oil by rapeseed oil in the diet resulted in slight increase in hepatic lipid content (Bell *et al.*, 2001). The reader should be aware that the above-mentioned studies have been performed on fish of different sizes and with different experimental durations, which might explain the differences observed.

*Fatty acid profile of whole body and tissues*

The substitution of fish oil by plant-derived oils as dietary lipid source in fish feed is highly correlated to a change of the fish fatty acid composition, mainly in the adipose tissue and fillet for fatty fish such as salmonids, and in the liver for lean fish such as Atlantic cod (Torstensen *et al.*, 2000; Lovell, 2003; Torstensen *et al.*, 2004a; Bell and Tocher, 2009; Turchini *et al.*, 2009).

Despite a relatively high capacity of salmonids to convert ALA to n-3 LC-PUFA, this endogenous production is not sufficient in fish fed plant-derived oil diets to maintain the EPA and DHA at similar levels as those reported in

wild fish or fish fed a fish oil diet. Consequently and as reported by numerous previous studies, the fish fatty acid composition after a feeding trial reflects that of the experimental diet administered. Therefore, the inclusion of plant-derived oils in fish diet highly reduces the n-3 LC-PUFA content in fish tissues whereas an increase in C18 PUFA content and a modification of the MUFA content from C20 and C22 MUFA to C18 MUFA are observed, such as in rainbow trout (Thanuthong *et al.*, 2011a; 2011b; Francis *et al.*, 2014; Yildiz *et al.*, 2014) and Atlantic salmon tissues (Torstensen *et al.*, 2000; Bell *et al.*, 2001; Tocher *et al.*, 2002; Zheng *et al.*, 2004b; Menoyo *et al.*, 2005; 2007; Leaver *et al.*, 2008b). Interestingly, the fatty acid profile of fish tissues is also impacted in terms of the fatty acid intermediates of the n-6 and n-3 PUFA bioconversion pathways (Rollin *et al.*, 2003b). In the fillet of rainbow trout, higher amounts of 18:4n-3, 20:3n-3 and 20:4n-3 were reported for fish fed a linseed oil diet, whereas higher amounts of 18:3n-6 and 20:3n-6 were reported for fish fed a sunflower oil diet, in comparison with fish fed a fish oil diet (Francis *et al.*, 2014). Similarly, in the liver of Atlantic salmon, increased amounts of 20:2n-6, 20:3n-6, 18:4n-3, 20:3n-3 and 20:4n-3 were observed for fish fed a linseed oil diet, as compared to fish fed a fish oil diet (Tocher *et al.*, 2003).

Turchini *et al.* (2011) estimated that the endogenous capacity of rainbow trout to produce EPA and DHA from dietary ALA could be interesting to reduce the n-3 LC-PUFA input/n-3 LC-PUFA output ratio in salmonid culture. However, this bioconversion cannot yet allow avoiding the decrease of the n-3 LC-PUFA fish content induced by the fish oil substitution. For example, despite an efficient fatty acid bioconversion capacity, a reduction from 5 to 15 % depending on the n-3 PUFA was observed in fillet and liver of rainbow trout fed a 100 % plant-based diet (Lazzarotto *et al.*, 2015). In Atlantic salmon, the replacement of fish oil by linseed oil in fish diet for 40 weeks induced a decrease of fillet EPA and DHA to 30 and 38 %, respectively, of the values reported in the fillet of fish fed a fish oil diet (Bell *et al.*, 2004).

The extent to which a dietary lipid source change may affect the fatty acid composition of fish tissues depends on the lipid class proportion. The fatty acid profile of the TAG class is highly impacted by the dietary lipid source, while the influence on the PL class is reduced (Menoyo *et al.*, 2005; Ruyter *et al.*, 2006; Turchini *et al.*, 2009). Therefore, the lipid content and the ratio between TAG and PL in the fish tissue modulate the impact of a dietary lipid

source replacement (Turchini *et al.*, 2009). The fillet fatty acid profile of salmonids is highly impacted by a dietary lipid source replacement as these fish species own a lipid-rich fillet with a high TAG content. Conversely, the impact of a dietary lipid source replacement is reduced on tissues of lean fish (*i.e.* cod, haddock and plaice) owning a low fillet lipid content with a low TAG/PL ratio (Bell and Tocher, 2009).

The influence of the dietary lipid source on fish fatty acid composition is logically increased with the dietary lipid content and depends also on the metabolic role of the specific fatty acids. Conversely to other fatty acids, a preferential deposition and retention of DHA is observed in the fillet of most of the fish species studied, irrespective of the dietary DHA concentration (Bell *et al.*, 2004; Bell and Tocher, 2009; Médale *et al.*, 2013). Among others, this phenomenon has been observed in salmonids fed plant-derived oil diets (Turchini *et al.*, 2009; Lazzarotto *et al.*, 2015).

Consequently to the dietary lipid source substitution, the intake of health promoting n-3 LC-PUFA brought by farmed fish to the human consumer is significantly reduced. However, despite this reduction in n-3 LC-PUFA in farmed salmonids fed with plant-derived oil feeds, the edible part of these animals still has a higher amount of n-3 LC-PUFA in comparison with the fillet of their wild counterparts and of some lean marine fish species, such as Atlantic cod (*Gadus morhua*) and sole (*Solea solea*) (Turchini *et al.*, 2011; Sprague *et al.*, 2016).

*Other criteria*

The dietary lipid source replacement of fish oil by alternative plant-derived oils may also modulate the fish organoleptic qualities, such as colour, flavour, texture and fillet stability (Turchini *et al.*, 2009; Hixson, 2014). The literature evaluating the dietary lipid source impacts on fish sensory quality is divided and contradictory. Interestingly, fillets from fish fed a 100 % plant-derived oil diet were preferred by a trained taste panel over those of fish fed a 100 % fish oil diet since fillets from fish fed plant-derived diets were less rancid and had less marine characteristics (Torstensen *et al.*, 2005).

Lazzarotto *et al.* (2015) studied the reproductive performance of rainbow trout after a complete cycle of breeding during which fish consumed a diet totally free of fish meal and fish oil. The authors observed an efficient fatty acid bioconversion and a selective fatty acid orientation since significant

amounts of AA, EPA and DHA were found in ova. Although the ova were smaller, trout fed a 100 % plant-based diet were able to produce ova and viable alevins (Lazzarotto *et al.*, 2015).

Studies on the effects of reduced dietary n-3 LC-PUFA content and increased dietary n-6 PUFA due to the dietary lipid source replacement on salmonid heart histology are contradictory (Bell *et al.*, 1993; Grisdale-Helland *et al.*, 2002; Turchini *et al.*, 2009). Detailed information is found in Turchini *et al.* (2009).

Recently, Francis and Turchini (2017) evaluated a new approach to increase the fish n-3 LC-PUFA retention through increased dietary protein content and therefore have 'retro-engineered' the protein sparing effect. Promising results have been obtained, regarding the significant sparing of SFA and MUFA, but this approach requires further in-depth investigation.

#### 1.3.2.2 Finishing diet use

The research is currently focussed on the development of feeding strategies aiming at producing farmed fish rich in n-3 LC-PUFA whilst minimising fish oil inclusion in fish feed. Finishing diets, given prior to harvest and formulated with high fish oil content, have been investigated to restore the n-3 LC-PUFA content and reduce the C18 PUFA content in the tissues of fish previously fed plant-based diets throughout the grow-out period (Corraze and Kaushik, 2009; Turchini *et al.*, 2009). Previous studies have demonstrated positive results in many fish species, including rainbow trout (Thanuthong *et al.*, 2011b; 2012; Francis *et al.*, 2014), Atlantic salmon (Bell *et al.*, 2003; Bell *et al.*, 2004), common carp (*Cyprinus carpio*) (Schultz *et al.*, 2015), red hybrid tilapia (*Oreochromis* sp.) (Ng *et al.*, 2013), European sea bass (*Dicentrarchus labrax*) (Mourente and Bell, 2006), red seabream (*Pagrus auratus*) (Glencross *et al.*, 2003) and Murray cod (*Maccullochella peelii peelii*) (Turchini *et al.*, 2006, 2007).

An EPA and DHA recovery rate of ~ 80 % was reported at the end of the 20-week finishing period in the flesh of Atlantic salmon previously fed diets formulated with linseed or rapeseed oils for 50 weeks, in comparison with fish fed a fish oil diet for 70 weeks. However, the LA content remained ~ 50 % higher than in fish fed the fish oil diet (Bell *et al.*, 2003). A similar recovery rate of ~ 80 % was reported for such feeding trial with Atlantic salmon fed a linseed oil diet for 40 weeks and then a fish oil diet for 24

weeks (Bell *et al.*, 2004). However, even if the finishing diets induced a shift in the fish fatty acid profile to a more fish oil-like composition, these were unable to achieve similar higher n-3 LC-PUFA and lower C18 PUFA levels (especially for LA) in fish as compared to fish fed fish oil throughout their growth period (Thanuthong *et al.*, 2011b). This was observed in rainbow trout fed a linseed, olive, palm or sunflower oil-based diet (Francis *et al.*, 2014) or a blend plant-derived oil diet (linseed and sunflower oils, 1:1) (Thanuthong *et al.*, 2012) and then a fish oil diet regarding the higher C18 PUFA content and lower n-3 LC-PUFA.

Numerous factors contribute to the efficiency of a finishing period. These include the fish species, the finishing period duration, the fatty acid profile of the alternative plant-derived oil used (Ng *et al.*, 2013; Francis *et al.*, 2014) (for example the dietary $C_{18}$ PUFA level (Thanuthong *et al.*, 2011b)), or the application of a short term feed deprivation period before the commencement of the finishing period (Thanuthong *et al.*, 2012). Interestingly, Turchini *et al.* (2006) observed that in Murray cod fed a canola or linseed oil diet, the accumulation of EPA and DHA in fillet during the finishing period using fish oil was faster than in fish continuously fed a fish oil diet. This study highlights the effectiveness of an optimisation process for the finishing period on the n-3 LC-PUFA content restoration in fish previously fed plant-derived oil diets.

The use of finishing diets is also effective regarding the enhanced growth observed for fish fed a plant-derived oil diet in comparison with fish fed a fish oil diet throughout. Turchini *et al.* (2007) reported a "lipo-compensatory growth" for Murray cod fed a plant-based diet and then a fish oil diet since these fish grew faster than those fed a fish oil diet continuously. This phenomenon has also been reported for Atlantic salmon when shifted from a rapeseed oil to a fish oil diet (Torstensen *et al.*, 2004b) and for red seabream fed a soybean oil diet and then a fish oil diet (Glencross *et al.*, 2003).

In conclusion, the different feeding strategies based on the use of a fish oil finishing diet demonstrate promising results and have undoubtedly positive environmental and economic effects. However, they still rely upon the inclusion of unsustainable dietary fish oil, while, on the other hand, the final fatty acid composition of the fillets is still not optimal.

#### 1.3.2.3 Early feeding

The nutritional conditioning during early life stages of fish may be an interesting feeding strategy to improve the lipid bioconversion capacity of juvenile and adult fish fed a ALA-rich plant-based diet (Vagner *et al.*, 2007; 2009; Geurden *et al.*, 2013).

In a study where rainbow trout fry from 1.2 to 13.1 g were fed a fish oil or plant-based diet containing deuterated ALA, a higher conversion of dietary deuterated ALA to DHA was observed for fish fed the plant-based diet, but, in addition, the bioconversion rate was higher in smaller fish from 0.5 to 1.5 g in comparison to larger fish from 6 to 8 g, highlighting the rapid change in bioconversion capacity with fish size (Bell and Dick, 2004). In European sea bass, an increased *fads2* gene expression was observed in juveniles fed an n-3 LC-PUFA deficient diet from day 83 post-hatch to day 118, when larvae had previously fed a low n-3 LC-PUFA diet (EPA + DHA 0.5 % DM) from day 6 to day 45, as compared to a high *n*-3 LC-PUFA diet (3.7 % DM) (Vagner *et al.*, 2009). Similarly, the *fads2* expression was increased in juvenile sea bass fed a low dietary LC-PUFA content at larval stage and then a LC-PUFA deprived diet at juvenile stage, despite a 3-month intermediate period using a commercial diet. Moreover, the increased *fads2* expression was correlated to increased DHA content in PL of fish previously fed a low LC-PUFA content as compared to those fed a high LC-PUFA content at larval stage (Vagner *et al.*, 2007). In seabream, a 6-fold increase in *fads2* mRNA level was reported in larvae fed diets in which fish oil was totally replaced by rapeseed or soybean oil. In contrast, larvae fed a linseed oil diet increased their *fads2* expression only by 2 fold in comparison with larvae fed a fish oil diet (Izquierdo *et al.*, 2008).

Considering the plant-based diet acceptance at juvenile stage, an increase in feed intake (30 %), FE (18 %) and growth rate (42 %) was reported for juvenile rainbow trout which had been fed a plant-based diet for 3 weeks at early-feeding fry stage, in comparison with a fish oil diet, even though both fish groups were subjected to a 7-month intermediate feeding period with a fish oil diet between the fry and juveniles stages (Geurden *et al.*, 2013).

### 1.3.3 Dietary modulator impact on fish lipid metabolism and physiology

#### 1.3.3.1 Introduction

The use of modulators added to fish feed could be an interesting approach to avoid the decrease or even to increase the n-3 LC-PUFA content of farmed fish. A few of such compounds have already been shown to affect the lipid metabolism in human and rodents. Moreover, different studies have also investigated their potential to enhance the bioconversion of dietary ALA in fish.

Iron is present in the active core of both Δ5 and Δ6 Fads2 and is essential for the introduction of a double bond into the aliphatic chain. In juvenile rainbow trout, the incorporation of iron in diets formulated with rapeseed and linseed oils led to an increase in the fillet n-3 LC-PUFA amount and in the desaturase activities without deleterious effects on growth (Senadheera *et al.*, 2012a). The pyridoxine (vitamin B6) also has a positive impact on the biosynthesis of LC-PUFA, as a cofactor or activator. Indeed, a dietary pyridoxine supplementation has been shown to promote the apparent *in vivo* elongase, Δ5 and Δ6 Fads2 activities in rainbow trout fed a rapeseed and linseed oil diet (Senadheera *et al.*, 2012b). In the same species, the inclusion of iron, zinc, magnesium, niacin, riboflavin, pyridoxine and biotin at 100, 200, 300 or 400 % of their recommended dietary inclusion seems to stimulate the fatty acid bioconversion of fish fed ALA-rich diets at the molecular and enzymatic level (Lewis *et al.*, 2013).

In Atlantic salmon, the effects of 3-thia fatty acids were investigated with the inclusion of dodecylthioacetic acid in a fish oil diet. A small increase in DHA and total n-3 PUFA was reported in the liver whereas the fish growth and the hepatic *pparα* gene expression were negatively impacted (Kleveland *et al.*, 2006). In rainbow trout, the dietary inclusion of the 3-thia fatty acid tetradecylthioacetic acid induced the increase of n-3 LC-PUFA in liver without negative effects on fish growth. However, the expression of the *fads2* was reduced whereas the elongases gene expression was increased (Kennedy *et al.*, 2007).

The fatty acid bioconversion capacity is also mediated by dietary conjugated LA (CLA, *i.e.* positional and geometric isomers of LA), which can have beneficial effects on human health (Belury, 2002). The inclusion of CLA to

the fish oil diet of rainbow trout induced a reduced *fads2* expression and an increased elongase gene expression (Kennedy *et al.*, 2007). Moreover, CLA was accumulated in flesh without detrimental effects on the n-3 LC-PUFA content (Kennedy *et al.*, 2007).

Fibrates are a class of lipid-regulating pharmaceuticals inducing hypolipidemic effects. As in mammals, these molecules are reported as Ppar ligands in fish (Ruyter *et al.*, 1997). Previous studies have reported that fibrates influence the fatty acid metabolism of salmonids through increase of peroxisomal β-oxidation (Ruyter *et al.*, 1997) and decrease of plasma lipoprotein concentration and n-3 LC-PUFA lipoprotein content (Prindiville *et al.*, 2011). In Atlantic salmon hepatocytes, the supplementation of fibrates induced increased *pparγ* expression and acyl-CoA oxidase activity (Ruyter *et al.*, 1997).

Even if cholesterol is not required by fish since they are able to synthesise it at the required concentrations, juvenile rainbow trout fed diets formulated with linseed and canola oils (70:30) showed an increased apparent *in vivo* activity of Δ6 Fads2 and elongases with increasing dietary cholesterol (Norambuena *et al.*, 2013).

Lignans, which are polyphenolic compounds found in plants, are also potential candidates as effective modulators of the lipid metabolism.

#### 1.3.3.2 Lignans

##### *1.3.3.2.1 Description and metabolism*

Lignans are a group of phenolic compounds widely distributed in the plant kingdom and commonly found in vascular plants. Plants have the ability to biosynthesise lignans from two C6-C3 coniferyl alcohol units present in the cell wall (Suzuki and Umezawa, 2007). Lignans are then usually deposited in wood, seeds or fruits in their free form or glycosylated to a variety of carbohydrates. Lignans are present in coniferous trees, seeds, nuts, cereals, vegetables, fruits, berries and different beverages. The highest concentrations in lignans are found in linseeds and sesame seeds (Manach *et al.*, 2004; Thompson *et al.*, 2006; Landete, 2012).

The secoisolariciresinol (SECO) and secoisolariciresinol diglucoside (SDG) belong to the dibenzylbutane subgroup of the "lignans with 9(9')-oxygen" category (Suzuki and Umezawa, 2007; Landete, 2012). SDG is the main

lignan of linseed and SECO corresponds to its de-conjugated form (Figure 1.7). Lignans are optically active and named by the direction in which they rotate the plane of polarised light ((+) or (-)) or by the spatial atom arrangement of the chiral centers (R or S). SECO exists in both enantiomeric forms R, R (+)-SECO and S, S (-)-SECO. In linseed, (+)-SECO is preferentially produced (Ford *et al.*, 2001). The SECO and SDG presented in Figure 1.7 are R, R (+)-SECO and R, R (+)-SDG.

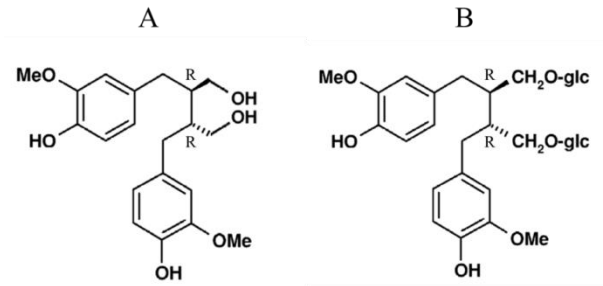


**Figure 1.7.** Structures and absolute configurations of (+)-secoisolariciresinol (A) and (+)-secoisolariciresinol diglucoside (B). Adapted from Wang (2002).

The sesamin (SES) and episesamin (EPI) belong to the furofuran subgroup of the "lignans with 9(9')-oxygen" category (Suzuki and Umezawa, 2007; Landete, 2012). They are called "sesame seed lignans" because these compounds are present in particularly high amounts in the sesame seed and its by-products. EPI, also called "asarinin", is not naturally present in seeds but is produced upon refining of the sesame seed oil through acid-catalysed epimerisation. The amount of SES transformed into EPI depends on the type of refined sesame oil: the proportion of converted SES can vary from 10 to 50 % (Li *et al.*, 2005; Moazzami *et al.*, 2007). The SES and EPI epimers are stereoisomers differing in their configuration on only one chiral center out of the four that these molecules possess. The SES presented in Figure 1.8 is S, R, R, S (+)-SES whereas the EPI is S, R, R, R (+)-EPI. As observed in Figure 1.8, SES exposes a symmetrical structure chair/chair in the plane whereas EPI has an asymmetric structure boat/chair. According to Li *et al.* (2005), the chair/chair conformation of SES is more stable than the boat/chair conformation of EPI.

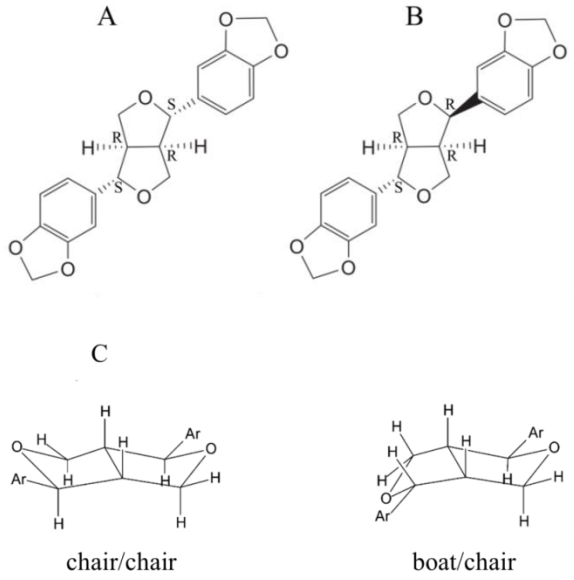


**Figure 1.8.** Structures and absolute configurations of (+)-sesamin (A) and (+)-episesamin (B). The sesamin conformation is termed chair/chair and the episesamin conformation is termed boat/chair (C). Ar, aromatic group. Adapted from Jin and Hattori (2011) and Li *et al.* (2005).

Linseed and sesame seed are the richest edible sources of lignans, which are often concentrated in the hull of the seeds (Landete, 2012). Over 3700 and 4000 μg of lignans have been quantified per g of linseed and sesame seed, respectively (Peñalvo *et al.*, 2005; Thompson *et al.*, 2006; Moazzami *et al.*, 2007). SES and EPI being more lipophilic than SECO and SDG, the amount of lignans recovered is therefore lower in linseed oil as compared to sesame seed oil (Peñalvo *et al.*, 2005).

In mammals, gastrointestinal bacteria are responsible for the conversion of plant lignans into the mammalian lignans enterodiol (END) and its oxidation product enterolactone (ENL). These compounds, also called enterolignans, are mainly produced and absorbed in the intestine and colon (Wang, 2002; Landete, 2012). The structures are presented in the absolute configurations R, R (+)-END and R, R (+)-ENL in Figure 1.9.

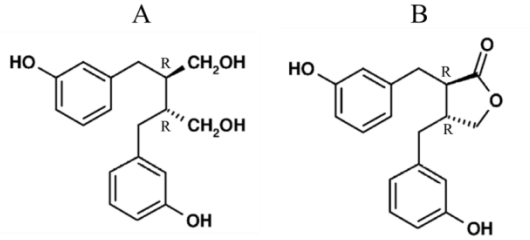


**Figure 1.9.** Structures and absolute configurations of (+)-enterodiol (A) and (+)-enterolactone (B). Adapted from Wang (2002).

In mammals, two conversion processes of the dietary plant lignans exist depending on the metabolised lignan. The transformation of SDG occurs in four steps. Firstly, the carbohydrate groups of SDG are hydrolysed by the gastrointestinal microflora to release SECO. This step is followed by a demethylation and a dehydroxylation by the colonic microbiota to produce END. Finally, END is oxidised to obtain ENL (Landete, 2012). SECO, END and ENL can then be absorbed, presumably through a passive diffusion and via epithelial transporters of the intestine and colon (Manach *et al.*, 2004).

The metabolism of dietary SES differs from the one of SDG. This lipophilic lignan is easily absorbed in intestine and colon in its native form and transported to the liver. In hepatocytes, SES is metabolised into hydroxylated metabolites, which are then excreted into the bile. In the gastrointestinal tract, the metabolites can be metabolised to END and ENL by the gastrointestinal microflora. END and ENL can then be absorbed and are transferred (Liu *et al.*, 2006; Landete, 2012). SES, as well as SDG and SECO, are considered as good mammalian lignan precursors (Liu *et al.*, 2006). Concerning the EPI metabolism, Jin & Hattori (2011) have also recorded EPI as a mammalian lignan precursor. No study has currently been focused on the metabolism of dietary plant lignans in fish. Although the fish intestine does not have a stable microflora (Neuman *et al.*, 2014), *Aeromonas*, *Pseudomonas* and the *Enterobacteriaceae* family are the most representative microorganisms of rainbow trout gastrointestinal tract (Huber *et al.*, 2004; Kim *et al.*, 2007). In comparison, among the wide mammalian microbiota, some genera such as *Peptostreptococcus*, *Eubacterium*, *Clostridium*, *Butyribacterium*, *Bacteroides*, *Ruminococcus* and *Bifidobacterium* are presumed to be involved in the conversion of plant lignans into END and ENL (Landete, 2012). The fish microflora appears therefore to differ from the one involved in the conversion of plant lignans in mammals. Further investigations should thus be carried out before drawing

any conclusion on the potential presence of endogenous enterolignans in the rainbow trout gastrointestinal tract.

Although they possess the same carbon skeleton, lignans display a broad structural diversity and therefore different biological properties. As most of the phenolic compounds, lignans are known to have a wide spectrum of health-promoting effects on human health. SDG, SECO, END and ENL are able to scavenge the hydroxyl and phenoxyl radicals and therefore exhibit an effective antioxidant effect against DNA damage, lipid peroxidation and other oxidative stress effects (Wang, 2002; Landete, 2012). They are presumed to contribute to reduction of hypercholesterolemia, hyperglycaemia, atherosclerosis and diabetes (Jeng and Hou, 2005; Landete, 2012). Concerning SES and EPI, only their catechol metabolites, END and ENL are considered to be effective antioxidants (Nakai *et al.*, 2003). Moreover, the mammalian lignans are one of the major phytoestrogen classes. Indeed, they are ligands of the oestrogen receptors, which are transcription factors of the nuclear receptor superfamily. These oestradiol-like compounds (high similarity with 17-β-oestradiol) can act as oestrogen agonists or antagonists, depending on the biological level of oestradiol. The END and ENL have been therefore highlighted to be protective against osteoporosis, menopausal syndrome, colon cancer development and to decrease the risk and the proliferation of breast and prostate cancers (Wang, 2002; Landete, 2012).

*1.3.3.2.2 Lignan impact on fish lipid metabolism and physiology*

In rats, the sesame seed lignans have been associated with increased activity and gene expression of enzymes involved in β-oxidation, decreased gene expression and activity of neosynthesis enzymes, reduced plasma and hepatic lipid and cholesterol levels, inhibition of Δ5 desaturation of n-6 PUFA but increased Δ5 desaturation of n-3 PUFA, through the activation of PPARα and the inhibition of SREBP1c (Fujiyama-Fujiwara *et al.*, 1995; Ashakumary *et al.*, 1999; Ide *et al.*, 2001; Kushiro *et al.*, 2002; Jeng and Hou, 2005; Kiso *et al.*, 2005; Lim *et al.*, 2007). These results have led to different studies investigating the potential capacity of these bioactive compounds to positively affect the fish lipid metabolism. Indeed, the incorporation of sesame seed lignans in aquaculture feeds was expected to increase the levels of EPA and DHA in farmed fish fed a plant-derived oil diet. However, the lipid metabolism and the fatty acid composition of the diet differ from salmonids to rats. To date, only SES and EPI have been

tested as potential modulators of the fatty acid bioconversion capacity of fish.

Depending on the study, the inclusion of sesame seed lignans in fish diets can have opposite effects on the fish growth performance. No effect was observed on the growth of rainbow trout (Trattner *et al.*, 2008a; Schiller Vestergren *et al.*, 2013), juvenile Atlantic salmon (Trattner *et al.*, 2011) and common carp (Mráz *et al.*, 2010). However, the growth was negatively affected in Atlantic salmon (Schiller Vestergren *et al.*, 2012) and in juvenile barramundi (*Lates calcacifer*) (Alhazzaa *et al.*, 2012).

Regarding the effects on the fatty acid composition, some apparent contradictions are present within the literature. The incorporation of an equimolar-mixture of SES and EPI in rainbow trout diets based on linseed oil or a blend of linseed and sunflower oils (6:4) increased the DHA proportion and decreased the LA, ALA, 20:4n-3 and total PUFA ones in the white muscle (Trattner *et al.*, 2008a). In contrast, the fatty acid composition of liver was not affected by the dietary lignan inclusion, which is positive in terms of fish welfare (Trattner *et al.*, 2008a). In a study on Atlantic salmon hepatocytes, the incubation of cells with SES and EPI for 48 h increased the elongation and desaturation of radioactive ALA into DHA and also the content in β-oxidation products (Trattner *et al.*, 2008b). In a second study on Atlantic salmon hepatocytes, the presence of SES and EPI increased the percentages of 18:4n-3 and 20:4n-3 but had no impact on the recovered EPA and DHA proportions (Schiller Vestergren *et al.*, 2011). The authors suggested that the discrepancy between their results and those of Trattner *et al.* (2008b) may be due to differences in the fish size (~ 800 g for Trattner *et al.* (2008b) against ~ 1300 g for Schiller Vestergren *et al.* (2011)) but also in the nutritional status of the fish used as source of hepatocytes for the primary cultures. In more recent studies, no major effect on the DHA percentage was detected in Atlantic salmon and rainbow trout tissues after a dietary sesame seed lignan inclusion (Trattner *et al.*, 2011; Schiller Vestergren *et al.*, 2012; 2013). However, a reduced ALA level was observed in rainbow trout fillet upon inclusion of SES in a linseed oil diet (Schiller Vestergren *et al.*, 2013) and in Atlantic salmon fillet upon SES and EPI inclusions in a plant-derived oil diet (Trattner *et al.*, 2011). Moreover, the SES inclusion in a diet mainly based on linseed oil decreased the amount of EPA and C22:5n-3 in Atlantic salmon white muscle and decreased the amount of C22:5n-3 in the liver (Schiller Vestergren *et al.*, 2012). The same study also demonstrated that the

SES inclusion in a diet mainly based on rapeseed oil decreased the amount of EPA and C22:5n-3 in Atlantic salmon liver.

The above mentioned studies have also evaluated the SES and EPI impacts on the expression of genes involved in the fatty acid β-oxidation, desaturation and elongation and also on the expression of transcription factors. Some discrepancies are also present between the studies. The expression of *pparα* in rainbow trout liver was down-regulated by the dietary inclusion of SES and EPI (Trattner *et al.*, 2008a) or by the inclusion of SES (Schiller Vestergren *et al.*, 2013) in a diet formulated with a blend of linseed and sunflower oils (6:4) for both studies. This result contrasts with the higher percentage of DHA and lower percentage of ALA observed in the fillet of rainbow trout (Trattner *et al.*, 2008a) since a down-regulation of *pparα* should induce a down-regulation of *fads2* expression. In contrast, an up-regulation of *pparα* expression was observed in Atlantic salmon fed a linseed and sunflower oil diet supplemented with SES and EPI inclusion (Trattner *et al.*, 2011). In Atlantic salmon hepatocytes, the incubation with sesame seed lignans decreased the expression of *pparα* and *fads2* whereas an increase of the desaturated products was observed (Trattner *et al.*, 2008b). The authors explained these discordant observations by higher translation rates of desaturases, as compared to their transcription rates. Conversely to the previous study, the incubation of Atlantic salmon hepatocytes with ALA, SES and EPI increased the expression of *pparα*, *elovl5*, *elovl2* and *fads2* while the incubation with SES alone showed no impact on the expression of the *pparα* gene (Schiller Vestergren *et al.*, 2011). Schiller Vestergren *et al.* (2012) studied the impact on the Atlantic salmon lipid metabolism of a SES inclusion in two different diets based on plant-derived oils. The diets differed in their n-6/n-3 fatty acid ratio (0.5 or 1). The expression of *pparα* was not affected for both diets. However, the transcription rates of the Δ5 and Δ6 desaturation genes and of the *elovl5* gene were enhanced by the SES addition to fish fed the low n-6/n-3 ratio diet. The increase of *srebp1* expression also observed with this diet could be responsible for the high transcription rate of the desaturation genes. Conversely, the addition of SES to the high n-6/n-3 ratio diet induced the opposite effect and down-regulated the expression of *fads2* and *elovl2*. These results highlight the various impacts of SES and EPI on salmonid lipid metabolism depending on the diet. The expression of other genes encoding proteins involved in the lipid metabolism, such as acyl CoA oxidase, carnitine palmitoyl-transferase I, scavenger receptor type B, cluster of differentiation 36, Elovl4, Srebp2,

Pparβ and Pparγ, has also been analysed. The data suggest additional regulatory effects by sesame seed lignans (Trattner *et al.*, 2008b; 2011; Schiller Vestergren *et al.*, 2012; 2013).

In contrast with human and rats where metabolites and enterolignans are the major compounds recovered, the sesame seed lignans are mainly found as such in rainbow trout muscles and liver (Trattner *et al.*, 2008a; 2011). Moreover, as for the rodent studies, the level of SES in rainbow trout tissues was found to be lower as compared to the EPI level (Trattner *et al.*, 2008a; 2011).

These results indicate that sesame seed lignans certainly impact the lipid metabolism of salmonids. The effects however appear to depend on various parameters such as the fish species and the dietary fatty acid composition. In addition, the mechanisms involved remain currently unclear.

## 1.4 Water temperature affecting fish lipid metabolism and physiology

### 1.4.1 Climate change and aquaculture production

The warming of the climate system is univocal and the increased atmosphere and water temperatures observed since the 1950s will significantly affect aquatic ecosystems. According to various climate models, climate change will be associated with a gradual rise of the earth surface temperature from 1 to 4°C by 2100 (IPCC, 2014). Since 1955, the global ocean has warmed substantially, mainly regarding the upper 700 m layer, inducing noticeable changes of fish marine ecosystems (IPCC, 2014). Freshwater resources are also strongly impacted by climate change. An increase of 0.2 to 2°C of water temperature has already been reported in lakes and rivers in Europe, North America and Asia (Cochrane *et al.*, 2009). The magnitude of temperature increase differs with the region concerned and appears to be correlated with latitude; the higher latitudes are predicted to suffer a larger temperature change than tropical and subtropical latitudes (Ficke *et al.*, 2007). Europe is one of the regions for which the largest increases in water temperature are projected (more than 2°C) (van Vliet *et al.*, 2013).

Climate change will have different effects on the marine ecosystems, including increased seawater temperature, increased seawater acidification, decreased oxygen level, ocean salinity variation (for example, a salinity decrease for Pacific whereas a salinity increase for Atlantic and Indian Oceans), increasing sea level and related factors such as changes in winds, strengths of storms and precipitation patterns, non-native species introduction and decrease in habitat and coastal zone quality (Cochrane *et al.*, 2009). Moreover, it is assumed that climate change may affect marine biodiversity by shifting species distribution, changing species community composition and reducing fish stock availability (Cochrane *et al.*, 2009). In freshwater systems, the impacts of changing water flows, the increase in flood and drought frequency and intensity and the human water use can be added to this list. The impacts are expected to be worse as compared to marine systems considering the reduced thermal inertia as compared to marine systems (Cochrane *et al.*, 2009; van Vliet *et al.*, 2013).

Climate change will directly and indirectly negatively impact the freshwater aquaculture systems regarding physiological, ecological and operational

aspects (Brander, 2007; Ficke *et al.*, 2007; Cochrane *et al.*, 2009; De Silva and Soto, 2009; Callaway *et al.*, 2012). These impacts include:

- stress due to increased temperature
- stress due to oxygen demand increase and declining oxygen water concentration
- uncertain supplies of freshwater (mostly for Africa and Asia)
- increase of extreme weather events, the precipitations being expected to increase globally and frequently in the form of extreme events
- sea level rise, which could cause an increase of salinity intrusion through rivers and thus impact the water supply quality of aquaculture farms
- increased frequency of diseases and toxic events via reduced fish immune function increased pathogens prevalence (particularly in temperate regions) and increased pollutant toxicity (for example organic and inorganic contaminants)
- spatial displacement of species specific aquaculture activities
- uncertain supply of fish meal and fish oil from capture fisheries, since marine fisheries, which will be impacted by the climate change, are a major source of inputs for aquaculture feed production

In contrast, some positive impacts of climate change on aquaculture production may appear and include: increased FE and growth rate for a temperature increase within the optimal growth temperature range, increased length of the growing season and range expansions polewards due to decrease in ice.

The freshwater salmonid farming sector is already subjected to increase water temperature in some temperate regions (van Vliet *et al.*, 2013). As compared to other fish species, rainbow trout possesses a narrow optimal range of temperature (between 9 and 16°C for an optimal growth). An increase of water temperature beyond 16°C, induced by the global warming, will therefore rapidly affect the growth performance of trout, through reduced FE, and thus its farming productivity. The salmonid farming is based on raceways or ponds with a free flow of water (partial in case of recirculating systems). Such water exchange could moderate the impacts of increased water temperature and reduced oxygen water level. However, the potential reduction in water supply and quality induced by climate change remains concerns that salmonid farming, as many other aquaculture production, will have to cope with (De Silva and Soto, 2009). In this context,

improvement and adaptation of the feeding practices are required (Cochrane *et al.*, 2009). Moreover, considering the energy costs of fish farming, the water temperature increase might also become problematic since the costs associated to water cooling are generally higher than those associated to water heating.

### 1.4.2 Water temperature increase impact on fish lipid metabolism and physiology

Temperature is one of the most important environmental factors affecting the metabolism and physiology of poikilothermic animals, such as fish. Changes in water temperature are directly linked to variations of fish biochemical rates and thus fish physiology, including homeostasis, growth, reproduction and activity (Ficke et al., 2007). The magnitude of the impact of an increased water temperature may differ with the fish species, the intraspecific variation within species, the previous life stage conditions, the fish size, the diet formulation, the dietary lipid content or the investigated tissues or lipid classes (Myrick and Cech Jr, 2000; Ficke *et al.*, 2007; Pörtner and Peck, 2010; Stitt *et al.*, 2014).

Considering the significant impact of increased water temperature on fish lipid metabolism and lipid composition, the relevant data reported in the literature are described below, with a special focus on salmonids.

*Digestion and absorption*

As mentioned in Section 1.2.2, the digestibility of a fatty acid is dependent on its melting point, the lower the melting point (corresponding to an higher unsaturation degree), the higher the apparent digestibility (Austreng *et al.*, 1980). It appears therefore that a change of water temperature highly impacts the digestibility through its influence on lipid fluidity, the effect of a temperature increase being more pronounced for the fatty acids with higher melting points (Huguet *et al.*, 2015).

In the study of Huguet *et al.* (2015) in which Atlantic salmon were fed varying AA/EPA ratios diets, the temperature increase from 10 to 20°C induced an increased apparent digestibility of dietary fatty acids, specifically of SFA, MUFA, 18:3n-6, and DHA whereas the apparent digestibility of n-6 PUFA (except 18:3n-6) and n-3 PUFA (except DHA) was not impacted. In rainbow trout fed crude palm oil-based diets, the temperature increase from

7 to 15°C increased the apparent digestibility of SFA, MUFA, LA, ALA, EPA and DHA (Ng *et al.*, 2003). In contrast, Austreng *et al.* (1980) reported no significant differences in lipid and fatty acid digestibilities in rainbow trout reared at 3 or 11°C. Conversely, in Atlantic salmon fed a soybean oil diet, the apparent digestibility of fatty acids was reduced with the increase of water temperature from 5 to 12°C (Grisdale-Helland *et al.*, 2002). Moreover, an increased water temperature of 5°C has been reported to reduce the apparent digestibility of MUFA, n-6 PUFA (particularly LA) and n-3 PUFA (particularly EPA and DHA) in rainbow trout fed a palm fatty acid distillate-based diet (FFA-rich oil), whereas the apparent digestibility of SFA was not affected (Ng *et al.*, 2010). The authors suggested that this reduction could be explained by a reduced lipase activity and/or an increased intestinal transit rate, reducing the contact time between lipases and dietary fatty acids (Ng *et al.*, 2010). A reduced lipase activity has also been reported for yellowtail kingfish (*Seriola lalandi*) when the rearing temperature was increased from 24 to 27°C (Bowyer *et al.*, 2014).

Considering the above-mentioned studies, the influence of water temperature on lipid and fatty acid digestibilities appears not to be conclusive. The differences between studies might be explained by the experimental temperature range selected and a different fish size used for the experiment. Windell *et al.* (1978) reported a significant decrease in lipid digestibility in rainbow trout of ~18 g reared at 7°C, as compared to fish reared at 11 or 15°C. However, this reduction in lipid digestibility was not observed in fish of ~ 207 g or ~ 586 g at the same temperature of 7°C. Moreover, it is worth noting that the impact of water temperature on fatty acid digestibility may be dependent of the dietary lipid source. In Atlantic salmon, the apparent digestibility of SFA was impacted by increased water temperature (5 or 12°C) for fish fed a 50 % soybean oil-based diet or a 100 % fish oil diet whereas no effect was reported for fish fed a 100 % soybean oil diet (Grisdale-Helland *et al.*, 2002).

*Transport and hepatic absorption*

The transport of lipids from enterocytes to the different tissues may be affected by the water temperature. For example, the amount of plasma lipoproteins in Atlantic salmon was observed to be reduced with the decreased sea water temperature from October (~ 8°C) to March (~ 4°C) (Torstensen *et al.*, 2004a).

In cultured hepatocytes from Atlantic salmon fed a soybean oil diet, an increased accumulation of radioactive ALA was recovered in hepatocytes incubated at 12°C and originated from fish held at 12°C, as compared to cells incubated and from fish held at 5°C (Ruyter *et al.*, 2003). This was explained by higher cellular metabolic rates, usually increasing with increased temperature. Moreover, the incorporation of radioactive ALA was higher than that of radioactive LA in hepatocytes incubated at 12°C, which was not observed at 5°C (Ruyter *et al.*, 2003). In contrast, in the same species, the uptake of OLA by cultured hepatocytes was lower for cells kept at 12°C, as compared to those at 5°C (Moya-Falcón *et al.*, 2006).

*Fatty acid mobilisation*

The water temperature exerts a major impact on the energy storage tissues of fish by increasing the energy demand associated to increased growth rate. In rainbow trout held under natural temperatures, the carnitine palmitoyl-transferase I activity in red fillet, and to a lesser extent in white fillet, was increased with the natural warmer temperatures in summer (Thibault *et al.*, 1997). In juvenile Atlantic salmon fed different dietary AA/EPA ratios, the apparent *in vivo* β-oxidation of SFA, MUFA, LA, ALA and EPA was increased at the higher water temperature of 20°C, as compared to 10°C (Norambuena *et al.*, 2015). Moreover, the hepatic expression of carnitine palmitoyl-transferase I (involved in mitochondrial β-oxidation) was increased with the increased temperature whereas the acyl-CoA oxidase (involved in peroxisomal β-oxidation) was not. Conversely, other studies reported that an increased water temperature was responsible for reduced β-oxidation in hepatocytes and enterocytes in fish (Tocher *et al.*, 2004; Moya-Falcón *et al.*, 2006). In cultured hepatocytes and enterocytes of rainbow trout fed a palm oil diet and reared at 7, 11 or 15°C, the increased temperature of 15°C induced a decreased fatty acid β-oxidation in both cell types (Tocher *et al.*, 2004). Moreover, an *in vitro* study on hepatocytes from Atlantic salmon fed a fish oil diet suggested that mitochondrial β-oxidation was lower at 12°C than 5°C (Moya-Falcón *et al.*, 2006).

*Neosynthesis*

In terms of water temperature impact on fatty acid neosynthesis, only one study has been found in the literature considering salmonids (Norambuena *et al.*, 2015). In that article, the increased water temperature of 20°C, as

compared to 10°C, reduced the hepatic expression of *fas* in juvenile Atlantic salmon fed diets differing in the AA/EPA ratio.

*Gene expression and enzymatic activity of desaturases, elongases and transcription factors*

An increase in water temperature has been several times reported to reduce desaturation and elongation activities in fish (Ninno *et al.*, 1974; Hagar and Hazel, 1985; Wodtke and Cossins, 1991; Tocher *et al.*, 2004; Vagner and Santigosa, 2011; Norambuena *et al.*, 2015). In Atlantic salmon fed diets with varying AA/EPA ratios, the high water temperature of 20°C induced a decrease of the apparent *in vivo* enzymatic activity of the Δ6 Fads2 and Elovl2, as compared to activities reported for fish reared at 10°C (Norambuena *et al.*, 2015). In contrast, the apparent *in vivo* activity of Elovl5 was not affected by the temperature, and no activity of the Δ5 Fads2 was detected at both temperatures. In rainbow trout, the transfer of fish from 5 to 20°C resulted in a transient, two days long, increased Δ6 Fads2 activity, which then returned to its initial level and remained constant (Hagar and Hazel, 1985). In contrast, no effect on Δ5 Fads2 was reported during the first days, while the activity declined from the third day. Tocher *et al.* (2004) observed reduced elongation and desaturation enzymatic activities in isolated hepatocytes and enterocytes from rainbow trout held at a water temperature of 15°C, in comparison with 7 and 11°C, and fed a palm oil-based diet. In cultured hepatocytes from Atlantic salmon fed a soybean oil diet, Ruyter *et al.* (2003) reported that the production rate of DHA from EPA was higher for fish held at 5°C as compared to 12°C, probably due to an increased elongation rate of 22:5n-3 to 24:5n-3 at the lower temperature.

The expression of genes related to the Δ5 and Δ6 Fads2 and to *elovl2* were significantly reduced at the increased temperature of 20°C, in comparison with 10°C, in Atlantic salmon fed diets with varying AA/EPA ratios, while the opposite was observed for the *elovl5* expression (Norambuena *et al.*, 2015). Concerning the impact of temperature on transcription factor expression, the increased temperature of 20°C induced an increase in the expression of *pparα* in Atlantic salmon fed diets containing different AA/EPA levels, in comparison with those reared at 10°C whereas the expressions of *pparβ* and *pparγ* were not impacted (Norambuena *et al.*, 2015).

*Growth performance*

It has been reported that rainbow trout energy requirements increase almost linearly with the water temperature up to an optimal level before decreasing, the feed intake is following a similar pattern, as observed in Figure 1.10 (Brett and Groves, 1979; Bureau *et al.*, 2003; Kestemont and Baras, 2007; NRC, 2011). The growth performance of fish is therefore highly impacted by the water temperature.

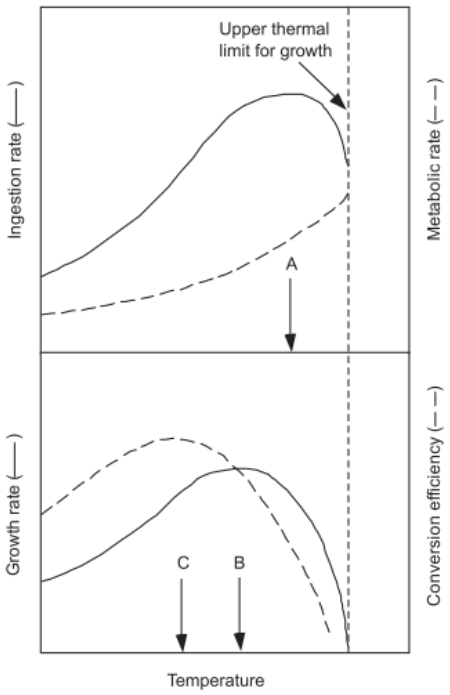


**Figure 1.10.** Temperature effect on feed intake, metabolism, growth and feed efficieny. The optimal temperature for maximum ingestion rate (A) is higher as compared to the optimal temperature for growth (B), which is higher as compared to the optimal temperature for efficient feed conversion (C) (Kestemont and Baras, 2007).

As a consequence of the increased energy requirements with increased water temperature, a higher feed intake and lower dietary protein and lipid retentions are generally reported for fish with temperature increase up to the thermal tolerance limit of fish (Abrahams, 2006; Callaway *et al.*, 2012). This has notably been observed in rainbow trout (Azevedo *et al.*, 1998) and Atlantic salmon (Grisdale-Helland *et al.*, 2002; Norambuena *et al.*, 2016). In

terms of FE, the values were not affected by increased water temperature in rainbow trout fed a fish oil diet (Azevedo *et al.*, 1998) or in Atlantic salmon fed a soybean oil diet (Grisdale-Helland *et al.*, 2002) whereas FE increased in Atlantic salmon raised at 20°C, as compared to 10°C, and fed diets with varying AA/EPA ratios (Norambuena *et al.*, 2016). Conversely, a reduced FE with temperature increase was reported for Atlantic salmon reared at 2 or 8°C and fed a plant-derived oil diet (Bendiksen *et al.*, 2003) and in Atlantic salmon held at 14 or 19°C and fed a fish and rapeseed oil blend diet (Hevrøy *et al.*, 2012). In Atlantic salmon, the temperature increase from 2 to 8°C induced enhanced growth of fish fed a plant-derived oil diet as the weight gain and the SGR were higher for fish held at 8°C (Bendiksen *et al.*, 2003). In contrast, a negative growth performance was reported for Atlantic salmon raised at 19°C in comparison with those at 14°C (Hevrøy *et al.*, 2012).

Decreasing HSI values are generally reported with temperature increase. For example, the HSI of Atlantic salmon reared at 12°C was reduced in comparison to fish held at 5°C (Grisdale-Helland *et al.*, 2002). This was also observed in Atlantic salmon fed diets with varying AA/EPA ratios and reared at 10 or 20°C (Norambuena *et al.*, 2016), in Atlantic salmon raised at 14 or 19°C and fed a plant-derived oil diet (Hevrøy *et al.*, 2012) or in rainbow trout acclimated at 5 or 20°C (Hazel, 1979).

Some contradictions with the above studies have been reported. For example, in Atlantic salmon post-smolts raised in seawater at 8°C, 12°C or 18°C for three months, reduced FE and HSI were observed with the temperature increase (Kullgren *et al.*, 2013). In contrast, however, a reduced feed intake was also reported. In another study, Hevrøy *et al.* (2012) fed adult Atlantic salmon a diet formulated with a blend of fish and rapeseed oils for 56 days. They found negative effects of an elevated temperature from 14 to 19°C on weight gain, FE and HSI but, again, feed intake was also negatively impacted (Hevrøy *et al.*, 2012). In juvenile rainbow trout subjected to a simulated global warming through a constant 2°C increase of water temperature in summer and winter, the growth rate, feed intake and FE were decreased in summer whereas an enhanced growth was reported in winter with the temperature increase (Morgan *et al.*, 2001). Interestingly, at summer water temperatures (11.6°C), an enhanced growth of Atlantic salmon was reported when 60 % of the dietary fish oil was replaced by rapeseed oil (Karalazos *et al.*, 2011). The authors suggested that this

increased growth was due to a higher digestibility of rapeseed oil, resulting in a higher energy supply for fish growth.

In the short term, the gradual increase of water temperature may lead to enhanced fish growth performance. However, as water temperature continues to increase with the global warming and because cultured fish species have a limited mobility in aquaculture, their productivity in aquaculture is likely to decline in the medium term (Cochrane *et al.*, 2009).

*Fish lipid content*

The water temperature impacts on the lipid content of whole body fish and its tissues. In rainbow trout fed a fish oil diet and reared at 6, 9, 12 and 15°C, the lipid content of whole body fish increased with the increased temperature (Azevedo *et al.*, 1998). In contrast, in Atlantic salmon fed a soybean oil diet, reduced lipid contents were reported in liver and intestine of fish reared at 12°C, as compared to those reared at 5°C (Ruyter *et al.*, 2006). Moreover, a reduced accumulation of intracellular lipid droplets was reported in the intestine of fish reared at 12°C. A lower lipid content was also reported in the liver of Atlantic salmon reared at the increased temperature of 20°C, in comparison with a water temperature of 10°C (Norambuena *et al.*, 2016). Interestingly, increased lipid content was observed with increased temperature (2 and 8°C) in fillet of Atlantic salmon fed a plant-derived oil diet with 20 % lipid content whereas the opposite (decreased fillet lipid content) was observed with the temperature increase for fish fed the plant-derived oil diet with 30 % lipid content (Jobling and Bendiksen, 2003).

*Membrane fatty acid composition*

In comparison with the NL class, the fatty acid composition of PL is highly dependent on the water temperature (Jobling and Bendiksen, 2003; Ruyter *et al.*, 2003). The cellular membranes, mainly based on PL, easily adapt to a water temperature change through their fluidity adaptation with PL, in order to maintain their functionality. A temperature increase is generally correlated to a decrease in the proportion of unsaturated fatty acids (MUFA and PUFA) and a corresponding increase in SFA proportion in membrane PL. Moreover, it has been observed that a cold acclimation was associated with increased proportions of phosphatidylethanolamine (PL with phosphoethanolamine as head group) and decreased proportions of phosphatidylcholine (PL with phosphocholine as head group) (Hazel, 1979, 1984; Cossins and Lee, 1985;

Hagar and Hazel, 1985; Fodor *et al.*, 1995; Tocher *et al.*, 2008). These changes are considered to be adaptive, in that they have an influence on the cellular metabolism of fish exposed to environmental temperature variations (Hazel, 1979; Cossins and Lee, 1985). A change in fatty acid composition of fish membranes due to a water temperature variation is termed "homeoviscous adaptation" (Hazel, 1984; Cossins and Lee, 1985).

In rainbow trout acclimated to 5 or 20°C, the increased temperature led to a decrease of PUFA, an increase of SFA and little change of MUFA content in hepatic PL (Hazel, 1979; Hagar and Hazel, 1985). In Atlantic salmon, the increase of water temperature from 5 to 12°C induced significant changes in fatty acid composition of hepatic PL as the SFA proportion increased whereas the MUFA proportion decreased. On the other hand, no effect was observed on n-6 and n-3 PUFA in hepatic PL (Ruyter *et al.*, 2006). In contrast, Jobling, Bendiksen (2003) observed only minor temperature effects on the PL fatty acid composition of Atlantic salmon fillet.

*Fatty acid profile of whole body and tissues*

The fatty acid composition of whole fish and its tissues are impacted by the water temperature. However, divergent results may be reported considering the tissue and the dietary lipid source.

Norambuena *et al.* (2016) reported significant effects on the fatty acid composition of Atlantic salmon and its tissues with the increase in water temperature from 10 to 20°C, and these effects varied according to the tissue. The accumulation of SFA, LA, AA and ALA was increased in the whole body with the increased temperature whereas the MUFA and n-3 LC-PUFA contents were not impacted. In the fillet, no effect of temperature was reported on the SFA, MUFA and n-6 PUFA contents whereas the n-3 PUFA were highly impacted with the decrease in ALA, EPA, 22:5n-3 and DHA contents. In contrast, in the liver, the SFA, AA, EPA, 22:5n-3 and DHA contents were increased with the temperature increase whereas the MUFA, LA, ALA contents decreased (Norambuena *et al.*, 2016).

In Atlantic salmon fed a soybean oil diet, the increased water temperature (from 5 to 12°C) had few effects on the fillet fatty acid composition, with the exception of slight increases in 16:0 and EPA (Grisdale-Helland *et al.*, 2002).

In steelhead trout fed a linseed oil diet, decreasing SFA and MUFA contents were observed in the fillet of fish raised at increasing temperatures from 10°C to 18°C while increasing ALA and DHA contents were observed and no effect was reported on the LA and AA, EPA contents (Wijekoon *et al.*, 2014). In contrast, in the same feeding trial with trout fed a sunflower oil diet, the increased temperature induced decreased fillet SFA and EPA contents and increased LA content and had no impact on the MUFA, ALA, AA and DHA contents (Wijekoon *et al.*, 2014).

*Finishing diet efficiency*

The efficiency of a finishing diet formulated with fish oil at the end of the grow-out period of fish fed a plant-derived oil diet throughout in order to increase their fillet n-3 LC-PUFA content has been proved (see Section 1.3.2.2). Interestingly, no influence of an increased water temperature from 15 to 20°C was reported by Codabaccus *et al.* (2013) on the finishing period efficiency when a fish oil diet was used with rainbow trout previously fed a 50 or 75 % palm fatty acid distillate diet.

### 1.4.3 Water temperature increase impact on fish physiology

*Physical and reproductive performance*

The swimming rate of fish may be negatively affected by the water temperature. In golden grey mullet (*Liza aurata*), fish reared at 20°C and fed a low n-3 LC-PUFA diet exhibited a higher expenditure of energy to reach the same maximum swimming speed as fish raised at 12°C on the same diet (Vagner *et al*., 2015). Moreover, brook trout acclimated at 20°C exhibited a lower critical swimming performance than fish acclimated to 12°C (Stitt *et al*., 2014). In contrast, no impact of temperature was reported on swimming performance of rainbow trout raised at 10, 14 or 19°C (Myrick and Cech Jr, 2000).

In addition to the effects of increased water temperature reported above, the cardiac tissues (Jørgensen *et al*., 2014), and the reproductive performance (Ficke *et al*., 2007; Donelson *et al*., 2014) are also affected by water temperature variations.

*Oxygen supply*

The oxygen water level is another environmental factor affecting the fish metabolism and its feeding behaviour (Kestemont and Baras, 2007). In case of water temperature increase, the biological oxygen demand of fish increases, such as observed in Atlantic salmon (Grisdale-Helland *et al*., 2002), according to the higher energy requirements. However, an increased water temperature also induces a reduced supply of dissolved oxygen (through reduced saturation concentrations relative to air) and thus negatively affects the fish metabolism (Ficke *et al*., 2007; Pörtner and Knust, 2007). In golden grey mullet, the increased temperature of 20°C induced a limited capacity of fish to increase their oxygen supply at the systemic level, as compared to fish raised at 2°C (Vagner *et al*., 2015).

## 1.5 Other environmental factors affecting fish lipid metabolism and physiology

The salinity effects on fish growth and bioconversion capacity have been particularly evaluated during the parr-smolt transformation of salmonids (Vagner and Santigosa, 2011). Generally, the *fads2* expression and Δ6 Fads2 enzymatic activity are higher in fresh water, especially around the seawater transfer, and decrease afterwards in sea water (Bell *et al.*, 1997; Tocher *et al.*, 2000; Zheng *et al.*, 2005b; Fonseca-Madrigal *et al.*, 2006; Vagner and Santigosa, 2011). Regarding the effect of salinity on growth of diadromous fish, results are not conclusive since positive, negative and no effects have been reported with seawater transfer (Kestemont and Baras, 2007).

Considering the impact of photoperiod, it has been reported in the literature that an increasing photoperiod stimulates the feeding activity. However, nothing is known about the regulation of fatty acid bioconversion capacity modulation by photoperiod (Vagner and Santigosa, 2011). However, Tocher *et al.* (2000) assumed that the increased desaturation and elongation enzymatic activities observed before the seawater transfer were modulated by the photoperiod, and also by the temperature and the diet.

## 1.6 Take-home messages

Rainbow trout has an optimal temperature range for growth between 9 and 16°C and supports temperatures up to 24°C. The juvenile and sub-adult rainbow trout requirements in PUFA can be covered if the diet contains around 1 % of LA (18:2n-6), and 1 % of ALA (18:3n-3) or 0.5 % of EPA (20:5n-3) and DHA (22:6n-3).

As other vertebrates, fish possess the capacity to biosynthesise n-3 LC-PUFA, such as EPA and DHA, from the precursor ALA, through desaturation and elongation. Similarly, the endogenous production of n-6 LC-PUFA, such as AA (20:4n-6), is derived from the precursor LA. As compared to other animals and fish species, salmonids possess a high fatty acid bioconversion capacity. The Δ5 and Δ6 Fads2, involved in the fatty acid desaturations, are derived from the *fads2* gene. The elongation steps are carried out by Elovl2 and Elovl5, which derive from the *elovl2* and *elovl5* genes, respectively. The dietary and endogenously produced fatty acids are mainly stored in perivisceral adipose tissue and fillet.

The production of salmonids with a high content in n-3 LC-PUFA is compromised with the unavoidable replacement of fish oil, rich in n-3 LC-PUFA, by alternative, often plant-derived, oils, lacking n-3 LC-PUFA. The replacement of fish oil by plant-derived oils in feed induces increased desaturation and elongation enzymatic activities in fish. An increased expression of *fads2*, *elovl2* and *elovl5* is also observed. The nutritional regulation of fatty acid metabolism is mediated notably through transcriptional factors (Lxr, Srebp, Ppar). Although generally not impacting fish growth, the inclusion of plant-derived oils in feed still results in fish with reduced n-3 LC-PUFA, which is detrimental in terms of fish health and fish benefits for humans. Feeding strategies are currently developed to counteract the fish n-3 LC-PUFA decrease. Promising results emerged from the finishing diet strategy and the early nutritional conditioning. Indeed, the use of finishing diets, given prior the harvest and based on fish oil, allows reducing the C18 PUFA content and increasing the n-3 LC-PUFA content of fish previously fed a plant-derived oil diet throughout the grow-out period. Moreover, the early conditioning of fish with n-3 LC-PUFA deficient diets showed to increase the *fads2* expression and positively impacted the feed intake and growth of fish at the sub-adult or adult stages. The dietary inclusion of bioactive compounds may also modulate the fish lipid metabolism. Among others, the sesame seed lignans have been reported to

affect the fish lipid metabolism, with both positive and negative effects reported.

Climate change will impact freshwater ecosystems and aquaculture systems through increased water temperature and decreased oxygen water concentration, among others. Fish being ectothermic animals, their metabolism is highly dependent on the temperature. Considering lipid metabolism, an increased water temperature induces decreasing fatty acid desaturation and elongation activities and gene expression. The fatty acid composition of cellular membranes is easily adapted to a temperature change, with generally increased SFA and decreased MUFA and PUFA with a temperature increase. On the short term, temperature increase is supposed to enhance fish growth performance, through increased feed intake. However, with the continuous water temperature increase, detrimental effects will emerge.

Even though some studies have previously evaluated the simultaneous impact of increased water temperature and dietary fish oil replacement on lipid metabolism of salmonids, to date, no study has investigated these effects on rainbow trout at both the whole fish and tissue levels, and with different complementary approaches. Moreover, even if promising methods exist, the development of feeding strategies is still required in order to produce fish with a high content in n-3 LC-PUFA while reducing the dietary fish oil inclusion. Such development will be efficiently performed with a thorough understanding of fish lipid metabolism and its potential nutritional modulation.

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# Chapter 2

## Aims of the thesis

The general purpose of the present work is to evaluate the impacts of temperature, fish n-3 PUFA depletion and lignans on the fatty acid bioconversion capacity of rainbow trout in a context of increased water temperature induced by the global warming and the unavoidable replacement of fish oil by alternative lipid sources into the fish feed.

More specifically, three specific goals have been defined:

1. **Evaluation of an increased water temperature on the fatty acid bioconversion capacity of rainbow trout fed a plant-derived diet**

In the near future, climate change is supposed to impact the freshwater aquaculture systems and fish physiology through pressures induced by, for instance, increased water temperature and reduced oxygen concentration. In this context, little is known about the impact of increased water temperature on the fatty acid bioconversion capacity of fish species, especially in a context of dietary fish oil replacement. In Chapter 3, we will evaluate the impact of an increased temperature on the growth, fatty acid composition and fatty acid metabolism of rainbow trout fed a plant-based diet.

An *in vivo* experiment will be conducted with rainbow trout fry at two different water temperatures: an optimal water temperature of 15°C and an increased water temperature of 19°C. Throughout the feeding trial, fish will be fed with a control diet formulated with fish oil or an experimental diet formulated with linseed oil (complete lipid source replacement). The fish growth and composition will be evaluated in addition to the fatty acid metabolism via the apparent *in vivo* whole body fatty acid balance method, as well as molecular analyses.

**2. Evaluation of the fatty acid bioconversion capacity of rainbow trout fry previously depleted in n-3 PUFA**

Research is currently carried out on the development of efficient and sustainable nutritional strategies to produce n-3 LC-PUFA-rich fish whilst replacing fish oil by plant-derived oils in aquafeeds. The development of such strategies requires a thorough understanding of fish fatty acid metabolism and its nutritional modulation. In Chapter 4, we will determine if the fatty acid bioconversion capacity of rainbow trout may be improved by previously depleting fish in n-3 PUFA.

Rainbow trout fry will be depleted in n-3 PUFA in a 60-day pre-experimental period by feeding a sunflower oil-based diet before to be fed with a fish oil-based diet or a linseed oil-based diet during a 36-day experimental period. The fatty acid bioconversion capacity of these fish will be compared to fish continuously fed a sunflower oil-, fish oil- or a linseed oil-based diet for 96 days. The impact of the fish n-3 PUFA depletion on the fish bioconversion capacity will be evaluated through fish fatty acid composition and the implementation of the whole body fatty acid balance method.

**3. Evaluation of the impact of lignans on the n-3 fatty acid biosynthesis pathway in the RTL-W1 cell line**

Some plant-derived oils are rich in lignans. Previous *in vivo* and *in vitro* studies reported favourable effects of the lignans SES and EPI from sesame seeds on the fatty acid bioconversion capacity of salmonids. In contrast, to date, no study has been carried out on the impact of lignans from linseed on fish lipid metabolism. However, a significant amount of lignans may be present in linseed oil diet and could affect the fish bioconversion capacity. In Chapter 5, we will assess the effects of sesame seed lignans, linseed lignans and enterolignans (potentially formed from dietary lignans through the gut microbiota) on the fatty acid bioconversion of the RTL-W1 cell line.

The sesame seed lignans SES and EPI, the linseed lignans SDG and SECO and the enterolignan END will be evaluated and compared *in vitro* as potential enzymatic modulators of the bioconversion of ALA into n-3 LC-PUFA in RTL-W1 cells enriched in ALA. The lignans and enterolignan will be supplemented to the culture medium of ALA-enriched cells for 48 h at 19°C and impacts will be evaluated on the cellular fatty acid composition.

# Chapter 3

## Temperature increase negatively affects the fatty acid bioconversion capacity of rainbow trout (*Oncorhynchus mykiss*) fed a linseed oil-based diet

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## Foreword

As presented in the Literature survey part, an increased water temperature may highly affect the fish lipid metabolism. However, few studies have evaluated the impact of a temperature increase on the fatty acid metabolism of fish concomitantly at the molecular, tissue composition and whole fish levels. Therefore, in the present chapter, we evaluated the influence of an increased temperature on growth, whole body fatty acid composition and fatty acid metabolism of fish fed a fish oil or a linseed oil diet. These results have been published in PloS ONE. Moreover, the fatty acid composition of fish fillet and adipose tissue has been determined. These data are presented and discussed as additional results. Finally, a description of the whole body fatty acid balance method used to calculate the apparent *in vivo* enzymatic activities is proposed as additional information.

## Abstract

Aquaculture is meant to provide fish rich in omega-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA). This objective must be reached despite (1) the necessity to replace the finite and limited fish oil in feed production and (2) the increased temperature of the supply water induced by the global warming. The objective of the present paper was to determine to what extent increased water temperature influences the fatty acid bioconversion capacity of rainbow trout (*Oncorhynchus mykiss*) fed a plant-derived diet. Fish were fed two diets formulated with fish oil (FO) or linseed oil (LO) as only added lipid source at the optimal water temperature of 15°C or at the increased water temperature of 19°C for 60 days. We observed that a temperature increase close to the upper limit of the species temperature tolerance range negatively affected the feed efficiency of rainbow trout fed LO despite a higher feed intake. The negative impact of increased water temperature on fatty acid bioconversion capacity appeared also to be quite clear considering the reduced expression of *fatty acid desaturase 2* in liver and intestine and the reduced Δ6 desaturase enzymatic activity in intestinal microsomes. The present results also highlighted a negative impact of increased temperature on the apparent *in vivo* enzymatic activity of Δ5 and Δ6 desaturases of fish fed LO. Interestingly, this last parameter appeared less affected than those mentioned above. This study highlights that the increased temperature that rainbow trout may face due to global warming could reduce their fatty acid bioconversion capacity. The unavoidable replacement of finite fish oil by more sustainable, readily available and economically viable alternative lipid sources in aquaculture feeds should take this undeniable environmental issue on aquaculture productivity into account.

## 3.1 Introduction

According to climate models, climate change will be associated with a gradual rise of surface temperature from 1 to 4°C by 2100 [1]. An increase of 0.2 to 2°C in water temperature has already been reported in lakes and rivers in Europe, North America and Asia [2]. This will affect freshwater fish communities and fisheries [3] and could impact directly and indirectly on aquaculture productivity [3, 4]. Increased water temperature is known to directly affect several physiological processes in fish, including growth [5, 6], basal metabolic rate [6, 7], digestive physiology [8, 9], swimming performance [10], cardiac function [11], reproductive performance [12], and oxidative stress management [13]. The detrimental effects of increased temperature on fish above optimum temperature for growth may be explained notably via its influence on biochemical reaction rates and via reduced oxygen availability and transport with increased temperature, which can therefore not respond to the higher tissue demand in oxygen [3, 14]. Moreover, tolerance to water temperature variation depends highly on fish species, previous life stage conditions and current physical resources [3, 10, 15]. Aquaculture productivity could also be affected indirectly by climate change through changes in precipitation patterns, river flow, drought frequency, increased pollutant toxicity and disease occurrences [1, 2, 4, 16]. In addition, aquaculture may suffer from reduced dietary ingredient availability, both in terms of fish meal and fish oil, resulting from a climate-associated impaired ocean productivity [2], and in terms of terrestrial ingredient alternatives, resulting from impaired crop production [1].

Fish oil is one of the most valuable ingredients used in fish feed production [4, 17, 18], owing to its lipid profile perfectly matching with salmonid fatty acid requirements [19, 20]. In addition, the use of fish oil in aquaculture enables farmed fish rich in omega-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA), namely eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), to be produced for the human consumer. The n-3 LC-PUFA are known to be involved in fundamental physiological processes and to have many positive effects on human health [21, 22]. As fish and seafood are the richest sources of n-3 LC-PUFA [21, 22] and these fatty acids are considered as semi-essential or essential for humans, the continuous supply of high nutritional value fish is of utmost importance. However, fish oil has become rare and expensive, economically and environmentally speaking, as it is a finite and limited marine resource

[17, 19]. This ingredient is thus progressively replaced by more sustainable alternative lipid sources in fish feed. In this context, plant-derived oils are considered as promising alternatives [18] and have already been included in commercial feeds without compromising fish growth performance [23-26]. Nevertheless, plant-derived oils do not contain n-3 LC-PUFA, which decreases the amount of EPA and DHA in the feeds and, consequently, the farmed fish, compromising their nutritional benefits to human consumers [18, 23-26]. Interestingly, although all plant-derived oils are devoid of n-3 LC-PUFA, a few such as linseed oil contain a high percentage of the n-3 LC-PUFA precursor, namely alpha-linolenic acid (ALA, 18:3n-3) [18, 19].

Since salmonid farming consumes about 60 % of the total fish oil used in commercial aquafeeds [27], research on fish oil replacement by plant-derived oils is particularly focused on these species. Among the salmonids, rainbow trout (*Oncorhynchus mykiss*) is an important cultured fish species in temperate regions [4, 17, 28]. Moreover, rainbow trout possesses a good capacity to endogenously produce EPA and DHA from ALA, via the n-3 fatty acyl desaturation and elongation pathway requiring Δ6 and Δ5 desaturases, and fatty acyl elongases (Figure 3.1), mainly in liver, intestine and brain [19, 29-31]. The endogenous production of arachidonic acid (ARA, 20:4n-6) from linoleic acid (LA, 18:2n-6) through the omega-6 (n-6) pathway takes place in parallel with the n-3 pathway utilising the same enzymatic system (Figure 3.1). The n-3 fatty acid bioconversion capacity of rainbow trout could therefore be exploited in order to continue providing the human consumer with fish rich in EPA and DHA, while replacing fish oil by linseed oil, rich in ALA, in feed. Promising results have already been reported [23-26], although the n-3 LC-PUFA content reported in these studies was lower than the n-3 LC-PUFA content of fish fed with fish oil. Interestingly, Tocher *et al.* [32] showed a significant LC-PUFA synthetic capacity in isolated hepatocytes of Atlantic salmon (*Salmo salar*) fed a plant-derived oil diet during its early growth stages, and the progressive decrease of that capacity until a size of 2 kg. Even at the early growth stage, the efficiency of bioconversion is of great interest from a nutritional point of view since it is during these days that the fish synthesise a significant part of their n-3 LC-PUFA from the corresponding precursors, in the case of permanent feeding with plant-derived oils, a large part of the newly synthesised n-3 LC-PUFA being expected to be kept in the fish body until a marketable size.

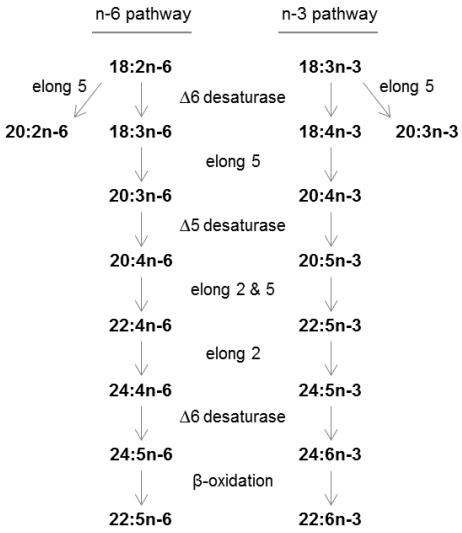


**Figure 3.1.** Pathways for endogenous elongation and desaturation of 18:2n-6 and 18:3n-3 to produce n-6 and n-3 long chain polyunsaturated fatty acids, respectively. The enzymatic system acts on both pathways in parallel requiring Δ6 and Δ5 desaturases and fatty acyl elongases 2 and 5. Elong, fatty acyl elongase.

Little is known about the impact of elevated water temperature on the fatty acid bioconversion capacity of rainbow trout. Rainbow trout possesses a very narrow optimal temperature growth range (15-16°C) but tolerates temperatures between 4°C and 20°C [28]. Increased water temperature in aquaculture will undoubtedly affect rainbow trout physiology and metabolism [4], and may thus modify its lipid bioconversion capacity, impacting the n-3 LC-PUFA content of fish fed plant-derived oils. Several studies have reported the combined influence of dietary lipid source and temperature in salmonids [33-37] and other fish species [5, 7, 38, 39]. Recent studies evaluating the impact of different ARA/EPA ratios and water temperature (10°C or 20°C) on Atlantic salmon growth and lipid metabolism concluded that increased temperature induced increased feed intake, hepatic ARA accumulation and apparent *in vivo* fatty acid β-oxidation. Conversely, decreased apparent *in vivo* activity of Δ6 desaturase and decreased expression of genes involved in fatty acyl desaturation (Δ5 and Δ6 desaturases) were observed [33, 34]. In contrast, Tocher *et al*. [35] observed reduced fatty acid β-oxidation, and lower elongation and desaturation enzymatic activities in isolated hepatocytes and enterocytes from rainbow

trout held at a water temperature of 15°C in comparison with 7 and 11°C, and fed a palm oil-based diet.

Considering the above, the objective of the present paper was to determine to what extent increased water temperature could influence the fatty acid bioconversion capacity of rainbow trout fed a plant-based diet by analysing fish growth, whole fish fatty acid composition, apparent *in vivo* enzymatic activities of desaturases and elongases determined by the whole body fatty acid balance method [40, 41], hepatic and intestinal gene expression and intestinal Δ6-desaturase enzymatic activity.

## 3.2 Experimental procedures

### 3.2.1 Ethics statement

The experimental design was approved by the Animal Care and Use Committee of the Université catholique de Louvain (Permit number: 123203) applying the EU legal frameworks relating to the protection of animals used for scientific purposes (Directive 86/609/CEE) and guidelines of Belgian legislation governing the ethical treatment of animals (Decree M.B. 05.01.1994, November 14$^{th}$, 1993). The feeding and digestibility trials were set up at the "Plateforme technologique en biologie aquicole Marcel Huet" (Université catholique de Louvain, Louvain-la-Neuve, Belgium), which is certified for animal services under permit number LA 1220034. All fish manipulations were performed under anaesthesia with 2-phenoxyethanol (Sigma-Aldrich, St Louis, MO, USA, 0.3 ml/l) and, if necessary, fish were killed with excess 2-phenoxyethanol. All efforts were made to minimise fish number and suffering. No clinical symptoms were observed within or outside the experimental periods.

### 3.2.2 Experimental diets

Two iso-energetic, iso-nitrogenous and iso-lipidic experimental diets were formulated to meet the nutrient requirements of rainbow trout [20]. Diets differed according to the added lipid source: the control diet (FO) was formulated with cod liver oil whereas the plant-derived diet (LO) was based on linseed oil. The detailed formulations and the proximate and fatty acid compositions are shown in Tables 3.1 and 3.2, respectively. The FO diet was particularly rich in n-3 LC-PUFA in which EPA (6.6 mg/g of dry matter (DM)) and DHA (10 mg/g DM) were the major fatty acids. In contrast, the

LO diet contained mainly ALA (38.6 mg/g DM) and residual levels of n-3 LC-PUFA (1.2 mg/g DM). Chromic oxide (Sigma-Aldrich) was added at 10 g/kg DM to each experimental diets intended for the digestibility trial in order to serve as indigestible marker. The experimental diets were produced as previously described [42]. Briefly, the dry components were homogenised (SM 20, Guangzhou Both-Win, Guangdong, China) before and after the oil addition and then after the water addition. After cold extrusion (HI 2251, Simplex, Paris, France) and freeze-drying, the diets were stored at -20°C until feeding or analysis.

**Table 3.1.** Components (g/kg of dry matter) and chemical composition of the control diet (FO) and the linseed oil diet (LO).

| | FO | LO |
|---|---|---|
| *Components* | | |
| Cod muscle meal [1] | 100.3 | 100.3 |
| Blood meal [2] | 130 | 130 |
| Wheat gluten meal [3] | 250 | 250 |
| Gelatin [4] | 50 | 50 |
| Lysine.HCl [4] | 10 | 10 |
| Modified starch [5] | 209.7 | 209.7 |
| Glucose [4] | 25 | 25 |
| Agar [4] | 10 | 10 |
| Carboxymethylcellulose [4] | 40 | 40 |
| Cellulose [4] | 10 | 10 |
| Cod liver oil [6] | 90 | 0 |
| Linseed oil [7] | 0 | 90 |
| Vitamin premix [8] | 10 | 10 |
| Mineral premix [9] | 65 | 65 |
| *Chemical composition* | | |
| Crude ash (% of DM) | 7.3 | 7.4 |
| Crude protein (% of DM) | 49.4 | 49.9 |
| Crude lipid (% of DM) | 10.8 | 11.5 |
| Gross energy (MJ/kg DM) | 21.5 | 21.6 |

DM, dry matter.

[1] Snick Euroingredients, Beernem, Belgium.

[2] Veos, Zwevezele, Belgium.

[3] Dumoulin, Seilles, Belgium.

[4] Sigma-Aldrich, St-Louis, MO, USA.

[5] Roquette, Lestrem, France.

[6] Certa, Waregem, Belgium.

[7] Eden Reform, Heimertingen, Germany.

[8] The vitamin complex (g/kg premix): retinol acetate 0.67, ascorbic acid 120, cholecalciferol 0.1, α-tocopherol acetate 34.2, menadione 2.2, thiamin 5.6, riboflavin 12, pyridoxine 4.5, calcium pantothenate 14.1, *p*-aminobenzoic acid 40, cyanocobalamin 0.03, niacin 30, biotin 0.1, choline chloride 350, folic acid 1.5, inositol 50, canthaxanthin 10, butylated hydroxytoluene 1.5, butylated hydroxyanisole 1.5, α-cellulose 322.1.

[9] The mineral complex (g/kg premix): $CaHPO_4.2H_2O$ 295.5, $Ca(H_2PO_4)_2.H_2O$ 217, $NaHCO_3$ 94.5, $Na_2SeO_3 \cdot 5H_2O$ 0.011, KCl 100, NaCl 172.4, KI 0.2, $MgCl_2$ 63.7, $MgSO_4.7H_2O$ 70.32, $MnSO_4.H_2O$ 1.52, $FeSO_4.7H_2O$ 12.41, $CuSO_4.5H_2O$ 0.4, $ZnSO_4.7H_2O$ 10.

**Table 3.2.** Fatty acid composition (mg/g of dry matter) of the control diet (FO) and the linseed oil diet (LO).

| Fatty acids | FO | LO |
|---|---|---|
| 14:0 | 3.0 | 0.1 |
| 16:0 | 11.5 | 6.5 |
| 18:0 | 2.4 | 3.6 |
| 16:1n-7 | 4.3 | 0.1 |
| 18:1n-7 | 3.1 | 0.9 |
| 18:1n-9 | 12.9 | 15.8 |
| 20:1n-9 | 6.2 | 0.0 |
| 18:2n-6 | 7.9 | 17.2 |
| 18:3n-6 | 0.1 | 0.0 |
| 20:2n-6 | 0.2 | 0.0 |
| 20:3n-6 | 0.1 | 0.0 |
| 20:4n-6 | 0.4 | 0.1 |
| 22:4n-6 | 0.0 | 0.0 |
| 22:5n-6 | 0.2 | 0.0 |
| 18:3n-3 | 1.1 | 38.6 |
| 18:4n-3 | 1.5 | 0.0 |
| 20:3n-3 | 0.2 | 0.0 |
| 20:4n-3 | 0.7 | 0.0 |
| 20:5n-3 | 6.6 | 0.3 |
| 22:5n-3 | 1 | 0.0 |
| 22:6n-3 | 10 | 0.8 |
| Total | 74.6 | 84.5 |
| Σ SFA [1] | 17.2 | 10.5 |
| Σ MUFA [2] | 27.4 | 16.9 |
| Σ C18 n-6 PUFA [3] | 8.0 | 17.2 |
| Σ n-6 LC-PUFA [4] | 0.9 | 0.2 |
| Σ C18 n-3 PUFA [5] | 2.6 | 38.6 |
| Σ n-3 LC-PUFA [6] | 18.5 | 1.2 |
| n-3/n-6 [7] | 2.4 | 2.3 |
| n-3 LC-PUFA /n-6 LC-PUFA [8] | 20.0 | 6.5 |

[1] Sum of saturated fatty acids, includes 20:0, 22:0 and 24:0.

[2] Sum of monounsaturated fatty acids, includes 14:1n-5, 22:1n-9 and 24:1n-9.

[3] Sum of omega-6 polyunsaturated fatty acids with 18 C.

[4] Sum of omega-6 long chain polyunsaturated fatty acids with 20 C and 22 C.

[5] Sum of omega-3 polyunsaturated fatty acids with 18C.

[6] Sum of omega-3 long chain polyunsaturated fatty acids with 20 C, 22 C and 24 C.

[7] Ratio of total omega-3 polyunsaturated fatty acids to total omega-6 polyunsaturated fatty acids.

[8] Ratio of omega-3 long chain polyunsaturated fatty acids to omega-6 long chain polyunsaturated fatty acids.

### 3.2.3 Fish and facilities

A feeding trial firstly provided data on growth performance, body proximate composition, fatty acid composition, and gene expression and desaturase activity in tissues of fish reared at an optimal growth temperature of 15°C or an increased temperature of 19°C and fed FO or LO. In addition, a digestibility trial was conducted at 19°C to evaluate the apparent digestibility of fatty acids in the experimental diets in order to apply the whole body fatty acid balance method.

Prior to the experiments, fish of domesticated origin (Pisciculture d'Hatrival, Hatrival, Belgium) were acclimatised at 12 ± 1°C and fed a commercial diet. The 60-day feeding trial was performed with rainbow trout of an initial mean weight of 8 g. After 48 h of feed deprivation, fish were randomly distributed among twenty tanks (55 l water volume) at a density of 40 fish per tank. Three additional tanks were used as initial condition and fish were anaesthetised directly after fish loading, weighed, dissected if necessary and kept frozen (-20°C) until homogenisation. Ten tanks were set at the optimal water temperature of 15.0 ± 0.9°C and ten tanks at the increased water temperature of 19.0 ± 0.5°C. Five tanks at each temperature were allocated to each dietary treatment ($n$ = 5 by experimental condition). The experiment duration was chosen to ensure a minimum tripling in body weight for all groups. Feeding was carried out by hand twice daily (09.00 and 17.00) to apparent satiation. The water was supplied at a 1 l/min flow and temperature was checked daily. Fish were subjected to a 12:12 h light:dark cycle photoperiod. Mortalities were recorded daily and dead fish removed. At the end of the feeding trial and after 48 h of feed deprivation, ten fish were weighed and stored together and seven more were weighed separately and dissected in order to collect the liver and intestine. Initial and final fish were freeze-dried, homogenised (Retsch, Haan, Allemagne) and stored at -20°C until analysis whereas liver and intestine were directly stored at -80°C after having been frozen in liquid nitrogen. The remaining fish from the feeding trial were brought together in one tank with a temperature of 12 ± 1°C and fed a commercial diet until the digestibility trial. The digestibility trial was performed on rainbow trout with an initial mean weight of 187 g. Each dietary treatment was applied to three circular tanks (130 l water volume) with 5.00 ± 0.05 kg of initial fresh fish body weight. The water temperature was maintained at 19.0 ± 0.3°C throughout the trial. Fish were subjected at a 12:12 h light:dark cycle photoperiod. After an adaptation feeding period of 3

days, the experiment was initiated and lasted 24 days in order to accumulate sufficient faeces. Fish were fed manually twice daily (09.00 and 17.00) to apparent satiation whilst avoiding any undesirable mixing of feed and faeces. The faeces were collected continuously through a rotating automatic faeces collector system [43]. The collected faeces were freeze-dried, homogenised and stored at -20 °C until further analysis.

### 3.2.4 Chemical analyses

The DM, crude ash, crude protein and crude lipid were analysed following analytical methods from the Association of Official Analytical Chemists [44] if not specified below. Briefly, DM and crude ash were measured by drying at 105°C for 16 h followed by an incineration at 550°C for 16 h. Crude protein was determined for diets after acid digestion with the Kjeldahl method (N × 6.25). Crude lipid was evaluated using diethyl ether extraction according to the Soxhlet method. Crude protein content of whole fish was determined as follows: crude protein (% DM) = 100 – crude ash (% DM) – crude lipid (% DM). Gross energy of diets was approximated as follows: gross energy (kJ/100 g DM) = crude protein (% DM) × 23.6 + crude lipid (% DM) × 39.5 + carbohydrates (% DM) × 17.2; where carbohydrates (% DM) = 100 - crude ash (% DM) - crude protein (% DM) - crude lipid (% DM) [20]. The chromium III (trivalent) concentration in diets and faeces was determined as described in [45]. Briefly, the protocol consisted in an acid digestion followed by an oxidation step and a spectrophotometric measurement (Cecil Instruments, Cambridge, UK) at 350 nm.

### 3.2.5 Performance parameters and fatty acid metabolism computation

Standard formulae were used to assess growth performance, feed utilisation and biometrical parameters throughout the feeding trial. These included initial and final weight, daily growth coefficient (DGC) expressed in ($g^{1/3}$/ day) x 1000 and calculated as follows : [(final body weight)$^{1/3}$ – (initial body weight)$^{1/3}$] x 1000/ (number of feeding days), thermal growth coefficient calculated as follows: [(final body weight) $^{1/3}$ – (initial body weight) $^{1/3}$ / temperature degree-days] x 1000, voluntary feed intake in g of dry feed/fish calculated as follows: $\sum_{i=0}^{t}$ (dry feed ingested at day *i* / number of fish at day *i*), t = number of feeding days, feed intake in %/day calculated as follows: [feed intake in g of dry feed/fish / (mean body weight x number of feeding days)] x 100, feed efficiency expressed in g/g of dry feed and

calculated as follows: (final body weight – initial body weight) / feed intake in g of dry feed/fish, protein efficiency ratio (PER) calculated as follows: (final body weight – initial body weight) / (nitrogen intake), nitrogen retention efficiency (NRE) expressed in % and calculated as follows: [(final body nitrogen – initial body nitrogen) / (nitrogen intake)] x 100, lipid efficiency ratio (LER) calculated as follows: (final body weight – initial body weight) / (lipid intake), lipid retention efficiency (LRE) expressed in % and calculated as follows: (final body lipid – initial body lipid) / (lipid intake) x 100, hepatosomatic index expressed in % and calculated as follows: (liver weight / body weight) x 100, intestinosomatic index expressed in % and calculated as follows: (intestine weight / body weight) x 100, and liposomatic index expressed in % and calculated as follows: (perivisceral lipid weight / body weight) x 100.

The estimation of the apparent *in vivo* fatty acid metabolism was calculated via the implementation of the whole body fatty acid balance method [40], with subsequent developments [41, 46]. Briefly, data relative to growth performance and feed intake, dietary and whole body fatty acid composition, and fatty acid digestibility were used in the computations required for the implementation of the method. The apparent fatty acid digestibility was assessed using the standard formula: 100 – [100 × ($Cr_2O_3$ in diet (mg/g DM)) / ($Cr_2O_3$ in faeces (mg/g DM)) × (fatty acid in faeces (mg/g DM)) / (fatty acid in diet (mg/g DM))]. The net appearance/disappearance of each fatty acid was determined as the difference between total fatty acid gain (= final fatty acid content - initial fatty acid content) and the net fatty acid intake (= total fatty acid intake - fatty acid egestion in faeces). The subsequent step involved a series of backwards computations along all the fatty acid bioconversion pathways [n-3 and n-6 polyunsaturated fatty acids (PUFA), saturated fatty acid (SFA) and monounsaturated fatty acids (MUFA)], as previously described in details [40, 41, 46]. Thanks to these calculations, the fate of each individual fatty acid towards bioconversion, oxidation or deposition, was therefore determined. Final results are reported as apparent *in vivo* enzymatic activity expressed as nmol per g of fish per day.

### 3.2.6 Fatty acid profile determination

For fatty acid profile determination of diets and whole fish, lipids were extracted following the method of Folch *et al.* [47] subsequently modified [46, 48]. Briefly, lipids of 1 g of dried sample were extracted by a mixture of chloroform/methanol (2:1, *v:v*) (VWR chemicals, Radnor, PA, USA).

Tridecanoic acid (Sigma-Aldrich) was used as internal standard for lipid quantification. The extracted fatty acids were converted into fatty acid methyl esters (FAME) via methylation in alkaline condition (KOH in methanol, 0.1 M, at 70°C for 60 min) and then in acid condition (HCl in methanol, 1.2 M, at 70°C for 20 min) and FAME subsequently separated by gas chromatography. The GC Trace (Thermo Scientific, Milan, Italy) was equipped with an RT2560 capillary column (100 m × 0.25 mm internal diameter, 0.2 µm film thickness) (Restek, Bellefonte, PA, USA), an automatic injector and a flame ionisation detector kept at a constant temperature of 255°C. The system used hydrogen as carrier gas at an operating pressure of 200 kPa. The oven temperature program was as follows: an initial temperature of 80°C, which progressively increased at 25°C/min up to 175°C, a holding temperature of 175°C during 25 min followed by an increase at 10°C/min up to 205°C, a holding temperature of 205°C during 4 min followed by an increase at 10 C/min up to 225°C and a holding temperature of 225°C during 20 min. Each peak was identified by comparison of retention times with those for pure methyl ester standards (Larodan and Nu-Check Prep, Elysian, USA). Data processing was via ChromQuest software 5.0 (Thermo Finnigan, Milan, Italy). The final results are expressed in mg/g of dry matter.

### 3.2.7 Tissue RNA extraction and quantitative realtime PCR (qPCR)

Gene expression was determined as previously described in Geay *et al.* [49]. Total RNA from approximately 100 mg of liver and intestine tissues was extracted using Extract-All® reagent (Eurobio, Courtaboeuf, France) followed by phase separation with chloroform and then precipitated with isopropanol. Based on the nucleic acid concentration measured by spectrophotometry (Nanodrop 2000c, NanoDrop Technologies, Wilmington, DE, USA), 20 µg of RNA was treated with the RTS DNase™ kit (MO BIO Laboratories, Carlsbad, CA, USA) in order to avoid genomic DNA contamination. RNA (1 µg) was then reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The relative expression of the *fatty acid desaturase 2* (*fads2)* and *elongase 5* (*elovl5)* genes was measured by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). PCR primers were designed according to the rainbow trout cDNA sequences for *fads2* and *elovl5* (Table 3.3). Amplification of the correct cDNA was confirmed by sequencing. The

elongation factor 1-α (*EF1α*) and *β-actin* gene expressions were verified not to be regulated by dietary treatment and temperature and were therefore used as reference genes to normalise the data (Table 3.3). Amplification of cDNA was carried out using the iQ$^{TM}$SYBR® Green Supermix (Bio-Rad Laboratories). Thermal cycling and fluorescence detection were conducted in a StepOnePlus Real-Time PCR System (Life technologies, Carlsbad, CA, USA) under the following conditions: 10 min of initial denaturation at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. After each run, amplification of single amplicon was confirmed by analysing the melt curve for each sample analysed in triplicate. Standard curves were performed for each primer set and primer efficiency (E) calculated as $E = 10^{(-1/slope)}$. The relative expression of *fads2* and e*lovl5* was obtained by normalising the mRNA levels of both genes to the geometric mean of *EF1α* and *β-actin* calculated with the relative standard curve method [50].

**Table 3.3.** Primers used for gene expression determination by quantitative real-time RT-PCR.

| Gene | Primer | Sequence primer (5'-3') | GenBank Acc. No. | Amplicon length |
|---|---|---|---|---|
| *fads2* | Forward | CGTCCTGGGAGACAAA CAGC | AF301910 | 256 bp |
| | Reverse | CTGATCAATGCTACGG AGCC | | |
| *elovl5* | Forward | CTATGGGCTCTCTGCT GTCC | AY605100 | 107 bp |
| | Reverse | TATCGTCTGGGACATG GTCA | | |
| *B-actin* | Forward | TTCAACCCTGCCATGT | AB196465 | 59 bp |
| | Reverse | ACGGCCAGAGGCGTA CAG | | |
| *EF1α* | Forward | ACCCTCCTCTTGGTCG TTTC | AF498320 | 64 bp |
| | Reverse | TGATGACACCAACAGC AACA | | |

*fads2*, fatty acid desaturase 2; *elovl5*, elongase 5; *EF1α*, elongation factor 1 alpha.

### 3.2.8 Δ6 Desaturation activity

The Δ6 desaturase enzymatic activity was performed on liver and intestine microsomes as previously described [49]. The tissue was homogenised in sucrose phosphate buffer (0.04 M, pH 7.4) containing 0.25 M sucrose, 0.15 M KCl, 40 mM KF and 1 mM *N*-acetylcysteine and then centrifuged at 25 000 g for 15 min in order to remove the fat upper layer and to collect the supernatant. The supernatant was then centrifuged at 105 000 g for 60 min at 4°C and the microsomal pellet was collected. The protein concentration of the microsomal pellet was determined using the Bio-Rad protein assay (Bio-Rad Laboratories) according to the Bradford dye-binding method [51]. Microsomes were incubated with 0.25 µCi of [1-$^{14}$C]18:3n-3 (Perkin Elmer, Waltham, MA, USA), added as a complex with fatty acid free- bovine serum albumin in phosphate-buffered saline, at 20°C for 1 h. The reaction was stopped with the first step of lipid extraction consisting in addition of chloroform/methanol (2:1, *v:v*) containing 0.01 % (w/v) butylated hydroxytoluene (BHT) as antioxidant. Lipids were transmethylated to FAME by acid-catalysed transesterification using toluene and 1 % $H_2SO_4$ in methanol overnight at 50°C. FAME were dissolved in 100 µl isohexane and applied as streaks on thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) previously coated with 2 g silver nitrate in 20 ml acetonitrile and activated at 110°C for 30 min. The TLC plates were fully developed in toluene/acetonitrile (95:5, *v:v*). Autoradiography was then performed with a Kodak MR2 film for seven days at room temperature. The area of silica containing the [1-$^{14}$C]18:4n-3 product was scraped into a scintillation mini-vial containing 2.5 ml of scintillation fluid (Meridian Biotechnologies, Epsom, UK) and the radioactivity was determined in a TRI-CARB 2000CA scintillation counter (United Technologies Packard, UK). Results were corrected for counting efficiency and quenching of $^{14}$C under these conditions and were expressed as pmol of [1-14C]18:4n-3 by hour and mg of microsomal protein. For technical reasons, no results could be obtained for the microsomes derived from liver samples of fish held at 15°C.

### 3.2.9 Statistical analysis

All the data are presented as mean ± SEM ($n$ = 4, 5 or 35, as stated). Effects of water temperature (T), dietary treatment (D), and water temperature × dietary treatment interaction (T × D) were analysed by a two-way analysis of variance (ANOVA), followed by Tukey's (parametric) or Student's

(nonparametric with α = 0.08 %) *post hoc* test in order to determine significant differences between conditions. Previously to statistical analysis, data were transformed with natural logarithm or square root if identified as non-homogenous (Levene's test) to meet the assumptions for statistical methods. Results of the two-way ANOVA test are reported in the Results section as: ns (not significant, $P > 0.05$), * ($P < 0.05$) or ** ($P < 0.01$). Statistical analysis was computed using JMP® Pro 11 (SAS, Cary, NC, USA).

## 3.3 Results

Each result section is presented considering firstly the temperature impact, then the dietary treatment impact, and finally the temperature and dietary treatment interaction impact if relevant. Two-way ANOVA and *post hoc* tests were used to compare the results and information is given in case of contradictory statistical results between both statistical tests.

### 3.3.1 Fish growth performance

The experimental conditions were readily accepted by fish and body weight increased by a minimum factor of six, as observed in Table 3.4. The mean mortality rate throughout the feeding trial was less than 0.1 % per day and was unrelated to the temperature or the diet.

**Temperature impact**

The increased temperature of 19°C induced no impact on fish weight and DGC but decreased the thermal growth coefficient for both dietary conditions ($P < 0.01$). Increased feed intake, expressed in % per day, was observed with increased temperature ($P < 0.01$), as highlighted by the *post hoc* statistical test, especially with fish fed LO. In contrast, the feed efficiency decreased for this condition ($P < 0.01$). More precisely, the temperature increase significantly reduced the feed conversion efficiency ratio and the retention efficiency of dietary proteins (PER and NRE, respectively) and lipids (LER and LRE, respectively) of fish fed LO, although the reduction of LRE was only shown by the ANOVA test. With both dietary treatments, the hepatosomatic and intestinosomatic indeces decreased with the temperature increase ($P < 0.01$).

**Table 3.4.** Growth performance, feed utilisation and biometrical parameters of rainbow trout reared at 15°C or 19°C on a control diet (FO) or a linseed oil diet (LO) over 60 feeding days.

| | 15°C | | 19°C | | | | |
|---|---|---|---|---|---|---|---|
| | FO | LO | FO | LO | T | D | T × D |
| *Growth performance and feed utilisation parameters* | | | | | | | |
| Initial weight (g/fish) | 8.17 ± 0.04 | 8.16 ± 0.08 | 7.92 ± 0.12 | 8.20 ± 0.04 | ns | ns | ns |
| Final weight (g/fish) | 54.35 ± 1.62 | 51.49 ± 1.70 | 53.41 ± 1.48 | 48.60 ± 1.11 | ns | * | ns |
| DGC (($g^{1/3}$/day) x 1000) | 29.54 ± 0.60 $^{a}$ | 28.43 ± 0.61 $^{ab}$ | 29.52 ± 0.50 $^{a}$ | 27.20 ± 0.51 $^{b}$ | ns | ** | ns |
| Thermal growth coefficient | 1.97 ± 0.04 $^{a}$ | 1.90 ± 0.04 $^{a}$ | 1.55 ± 0.03 $^{b}$ | 1.43 ± 0.03 $^{c}$ | ** | ** | ns |
| Feed intake in g of dry feed/fish | 39.23 ± 1.13 | 36.81 ± 0.94 | 40.55 ± 1.20 | 37.86 ± 1.36 | ns | * | ns |
| Feed intake in %/ day | 2.09 ± 0.05 $^{ab}$ | 2.06 ± 0.02 $^{b}$ | 2.20 ± 0.03 $^{ab}$ | 2.22 ± 0.04 $^{a}$ | ** | ns | ns |
| Feed efficiency (g/g of dry feed) | 1.18 ± 0.03 $^{a}$ | 1.18 ± 0.02 $^{a}$ | 1.12 ± 0.01 $^{ab}$ | 1.07 ± 0.01 $^{b}$ | ** | ns | ns |
| PER | 2.39 ± 0.06 $^{a}$ | 2.36 ± 0.04 $^{a}$ | 2.27 ± 0.03 $^{ab}$ | 2.14 ± 0.03 $^{b}$ | ** | ns | ns |
| NRE (%) | 37.13 ± 1.01 $^{ab}$ | 38.39 ± 0.69 $^{a}$ | 37.74 ± 0.55 $^{a}$ | 34.53 ± 0.15 $^{b}$ | * | ns | ** |
| LER | 10.91 ± 0.28 $^{a}$ | 10.20 ± 0.19 $^{a}$ | 10.39 ± 0.12 $^{a}$ | 9.27 ± 0.11 $^{b}$ | ** | ** | ns |
| LRE (%) | 96.60 ± 3.13 $^{a}$ | 80.24 ± 2.11 $^{bc}$ | 86.22 ± 1.34 $^{b}$ | 76.17 ± 1.32 $^{c}$ | ** | ** | ns |
| *Biometrical parameters* | | | | | | | |
| Hepatosomatic index (%) | 1.39 ± 0.04 $^{a}$ | 1.48 ± 0.03 $^{a}$ | 1.04 ± 0.03 $^{b}$ | 1.10 ± 0.03 $^{b}$ | ** | * | ns |
| Intestinosomatic index (%) | 3.23 ± 0.08 $^{a}$ | 3.36 ± 0.1 $^{a}$ | 2.76 ± 0.08 $^{b}$ | 2.84 ± 0.07 $^{b}$ | ** | ns | ns |
| Liposomatic index (%) | 1.51 ± 0.08 | 1.7 ± 0.09 | 1.49 ± 0.09 | 1.67 ± 0.08 | ns | * | ns |

Mean values (± SEM) within a row with no common superscript letter are significantly different (Tukey's *post hoc* test on log transformed values). P values relative to two-way ANOVA are reported in the last three columns of the table (*, $P < 0.05$; **, $P < 0.01$; ns, $P > 0.05$; $n = 5$ or hepatosomatic, intestinosomatic and liposomatic indeces: $n = 35$). DGC, daily growth coefficient; PER, protein efficiency ratio; NRE, nitrogen retention efficiency; LER, lipid efficiency ratio; LRE, lipid retention efficiency.

**Diet impact**

Considering fish held at 19°C, growth was negatively impacted by the reduced DGC and thermal growth coefficient ($P < 0.01$) for fish fed LO compared to FO. In contrast, fish growth was not impacted by dietary treatment in fish held at 15°C. Feed intake, expressed in % per day, and feed efficiency were unrelated to diet ($P > 0.05$). However, at the increased temperature of 19°C, NRE and dietary lipid conversion and retention, LER and LRE respectively, decreased in fish fed LO compared to fish fed FO. Slight increases in the hepatosomatic and liposomatic indeces were recorded in fish fed LO ($P < 0.05$), although these were not highlighted by the *post hoc* test.

### 3.3.2 Fish proximate composition

**Temperature impact**

No impact of temperature was observed on whole fish proximate composition, as observed in Table 3.5, although ANOVA revealed a temperature effect on crude protein content.

**Diet impact**

The dietary treatment did not affect whole fish proximate composition, with the exception of reduced crude lipid content of fish fed LO compared to fish fed FO at 15°C ($P < 0.01$).

**Table 3.5.** Initial and final proximate composition (mg/g of wet matter) of rainbow trout subjected to a feeding trial at 15°C or 19°C with a control diet (FO) or a linseed oil diet (LO).

| | | 15°C | | 19°C | | | | |
|---|---|---|---|---|---|---|---|---|
| | Initial | FO | LO | FO | LO | T | D | T × D |
| Dry matter | 241.7 ± 5.1 | 264.4 ± 1.3 | 261.4 ± 1.4 | 269.7 ± 1.6 | 264.4 ± 3.5 | ns | ns | ns |
| Crude ash | 21.0 ± 0.7 | 23.8 ± 0.4 | 23.3 ± 0.5 | 24.7 ± 1.1 | 24.7 ± 0.6 | ns | ns | ns |
| Crude lipid | 79.3 ± 0.8 | 87.1 ± 1.5 [a] | 78.8 ± 0.9 [b] | 82.4 ± 0.9 [ab] | 81.8 ± 1.9 [ab] | ns | ** | * |
| Crude protein | 141.3 ± 3.7 | 153.5 ± 0.7 | 159.3 ± 0.4 | 162.5 ± 2.2 | 157.8 ± 1.7 | * | ns | ** |

Mean values (± SEM) within a row with no common superscript letter are significantly different (Tukey's *post hoc* test on log transformed final condition values). P values relative to two-way ANOVA are reported in the last three columns of the table (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; $n = 5$).

### 3.3.3 Whole body fatty acid composition

**Temperature impact**

The fatty acid composition of whole trout is presented in Table 3.6. No effect of temperature was recorded on the SFA and MUFA contents. Regarding fish fed LO, the temperature increase induced increased C18 n-6 PUFA content (mainly LA) and a slight decrease in n-6 LC-PUFA content ($P < 0.05$). Regarding the n-3 family, more C18 n-3 PUFA and less n-3 LC-PUFA were observed at increased temperature. This last effect was however detected by the ANOVA test ($P < 0.01$) but not by the *post hoc* test. The ALA content was higher for fish raised at 19°C compared to 15°C, especially with fish fed LO, whereas the ANOVA statistical test highlighted the opposite effect on 20:4n-3 and EPA contents ($P < 0.01$). Concerning fish fed FO, lower DHA content was recorded with increased temperature. The n-3/n-6 ratio was slightly reduced with the temperature increase ($P < 0.05$) whereas the n-3 LC-PUFA /n-6 LC-PUFA ratio was not impacted.

**Diet impact**

Decreased SFA and MUFA contents were observed for fish fed LO (Table 3.6). In contrast, an increase in LA and ALA and their desaturation products, 18:3n-6 and 18:4n-3 respectively, was observed with the replacement of FO by LO. The n-6 LC-PUFA content slightly increased upon feeding fish with LO mainly due to a significant increase in 20:3n-6 ($P < 0.01$), although the n-6 LC-PUFA, ARA and 22:4n-6, decreased. The level of n-3 LC-PUFA was reduced in fish fed LO ($P < 0.01$). This was due to decreased contents of EPA, 22:5n-3 and DHA. Amounts of 20:3n-3 and 20:4n-3 increased however in the presence of the ALA precursor in fish fed LO. Overall, the lipid source replacement led to an increased n-3/n-6 ratio whereas the n-3 LC-PUFA /n-6 LC-PUFA ratio decreased for fish fed LO ($P < 0.01$).

**Temperature × Diet impact**

A temperature × diet interaction was observed on C18 n-6 PUFA, specially the LA content, as no effect of temperature was observed in fish fed FO whereas fish fed LO exhibited an increase in these contents with increased temperature ($P < 0.05$).

**Table 3.6.** Initial and final whole body fatty acid profile (mg/g of dry matter) of rainbow trout subjected to a feeding trial at 15°C or 19°C with a control diet (FO) or a linseed oil diet (LO) [1].

| | | 15°C | | 19°C | | | | |
|---|---|---|---|---|---|---|---|---|
| Fatty acids | Initial | FO | LO | FO | LO | T | D | T × D |
| 18:2n-6 | 18.77 ± 0.50 | 19.85 ± 0.22 [c] | 29.89 ± 0.61 [b] | 19.71 ± 0.20 [c] | 32.42 ± 0.74 [a] | * | ** | * |
| 18:3n-6 | 0.66 ± 0.01 | 0.47 ± 0.01 [b] | 0.84 ± 0.02 [a] | 0.40 ± 0.01 [c] | 0.80 ± 0.02 [a] | ** | ** | ns |
| 20:2n-6 | 1.08 ± 0.04 | 1.48 ± 0.02 | 1.62 ± 0.08 | 1.38 ± 0.02 | 1.66 ± 0.07 | ns | ** | ns |
| 20:3n-6 | 0.89 ± 0.03 | 0.93 ± 0.01 [b] | 1.47 ± 0.05 [a] | 0.78 ± 0.01 [c] | 1.30 ± 0.03 [a] | ** | ** | ns |
| 20:4n-6 | 2.45 ± 0.07 | 1.27 ± 0.02 [a] | 1.07 ± 0.03 [b] | 1.23 ± 0.02 [a] | 1.00 ± 0.03 [b] | ns | ** | ns |
| 22:4n-6 | 0.24 ± 0.00 | 0.13 ± 0.01 [a] | 0.1 ± 0.00 [b] | 0.13 ± 0.01 [a] | 0.09 ± 0.01 [b] | ns | ** | ns |
| 22:5n-6 | 0.72 ± 0.06 | 0.44 ± 0.03 | 0.36 ± 0.04 | 0.45 ± 0.02 | 0.37 ± 0.02 | ns | ** | ns |
| 18:3n-3 | 2.94 ± 0.02 | 2.28 ± 0.08 [c] | 36.81 ± 1.02 [b] | 2.67 ± 0.10 [c] | 40.39 ± 1.10 [a] | ** | ** | ns |
| 18:4n-3 | 4.07 ± 0.04 | 1.95 ± 0.03 [b] | 4.47 ± 0.06 [a] | 1.91 ± 0.02 [b] | 4.47 ± 0.09 [a] | ns | ** | ns |
| 20:3n-3 | 0.48 ± 0.03 | 0.45 ± 0.03 [b] | 2.83 ± 0.13 [a] | 0.51 ± 0.03 [b] | 2.87 ± 0.14 [a] | ns | ** | ns |
| 20:4n-3 | 2.87 ± 0.09 | 1.48 ± 0.04 [b] | 2.49 ± 0.18 [a] | 1.30 ± 0.01 [b] | 2.13 ± 0.08 [a] | ** | ** | ns |
| 20:5n-3 | 21.17 ± 0.34 | 7.75 ± 0.14 [a] | 4.42 ± 0.13 [b] | 7.34 ± 0.16 [a] | 4.11 ± 0.04 [b] | ** | ** | ns |
| 22:5n-3 | 6.44 ± 0.21 | 2.57 ± 0.05 [a] | 1.56 ± 0.09 [b] | 2.59 ± 0.05 [a] | 1.63 ± 0.01 [b] | ns | ** | ns |
| 24:5n-3 | 1.11 ± 0.06 | 0.62 ± 0.04 [a] | 0.22 ± 0.03 [b] | 0.56 ± 0.01 [a] | 0.23 ± 0.01 [b] | ns | ** | ns |
| 24:6n-3 | 1.52 ± 0.02 | 0.85 ± 0.03 [a] | 0.46 ± 0.01 [b] | 0.85 ± 0.02 [a] | 0.51 ± 0.02 [b] | ns | ** | ns |
| 22:6n-3 | 41.61 ± 0.98 | 25.03 ± 0.35 [a] | 14.65 ± 0.33 [c] | 23.73 ± 0.23 [b] | 14.02 ± 0.21 [c] | ** | ** | ns |

**Table 3.6** - Continued

| | | 15°C | | 19°C | | | | |
|---|---|---|---|---|---|---|---|---|
| Fatty acids | Initial | FO | LO | FO | LO | T | D | T × D |
| Σ SFA | 81.77 ± 1.70 | 80.98 ± 0.55 [a] | 63.42 ± 1.70 [b] | 76.75 ± 1.08 [a] | 67.68 ± 2.04 [b] | ns | ** | * |
| Σ MUFA | 81.77 ± 1.54 | 122.48 ± 0.38 [a] | 94.88 ± 3.11 [c] | 112.18 ± 1.62 [b] | 98.68 ± 3.29 [c] | ns | ** | * |
| Σ C18 n-6 PUFA | 19.42 ± 0.51 | 20.32 ± 0.22 [c] | 30.73 ± 0.61 [b] | 20.11 ± 0.20 [c] | 33.22 ± 0.76 [a] | * | ** | * |
| Σ n-6 LC-PUFA | 5.38 ± 0.18 | 4.24 ± 0.06 [ab] | 4.62 ± 0.09 [a] | 3.97 ± 0.03 [b] | 4.42 ± 0.14 [ab] | * | ** | ns |
| Σ C18 n-3 PUFA | 7.01 ± 0.06 | 4.23 ± 0.09 [c] | 41.28 ± 1.06 [b] | 4.58 ± 0.10 [c] | 44.86 ± 1.15 [a] | ** | ** | ns |
| Σ n-3 LC-PUFA | 75.22 ± 1.71 | 38.75 ± 0.51 [a] | 26.62 ± 0.63 [b] | 36.87 ± 0.33 [a] | 25.49 ± 0.40 [b] | ** | ** | ns |
| n-3/n-6 | 3.32 ± 0.02 | 1.75 ± 0.00 [b] | 1.92 ± 0.03 [a] | 1.72 ± 0.02 [b] | 1.87 ± 0.01 [a] | * | ** | ns |
| n-3 LC-PUFA /n-6 LC-PUFA | 14.00 ± 0.18 | 9.14 ± 0.07 [a] | 5.77 ± 0.22 [b] | 9.29 ± 0.11 [a] | 5.78 ± 0.11 [b] | ns | ** | ns |
| Total | 270.66 ± 5.58 | 271.00 ± 1.32 [a] | 261.55 ± 5.59 [a] | 254.49 ± 2.76 [a] | 274.36 ± 7.36 [a] | ns | ns | ** |

Mean values (± SEM) within a row with no common superscript letter are significantly different (Tukey's (parametric) or Student's (nonparametric with α = 0.8 %) *post hoc* tests on square root transformed final condition values). P values relative to two-way ANOVA are reported in the last three columns of the table (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; $n = 5$ except for LO at 15°C for which $n = 4$).

[1] See Table 3.2 for abbreviations.

### 3.3.4 Apparent *in vivo* fatty acid bioconversion

## Fatty acid appearance and disappearance

### Temperature impact

As observed in Table 3.7, the n-6 pathway bioconversion capacity seemed to be affected by the temperature increase with reduced appearances of some intermediates such as 18:3n-6, 20:3n-6 and ARA ($P < 0.05$). The lower appearances were nevertheless not significant as highlighted by the *post hoc* tests. Regarding the n-3 family, the ALA disappearance was higher in fish fed LO at increased temperature ($P < 0.05$). While 18:4n-3 and 20:3n-3 were not impacted by temperature, 20:4n-3, EPA and DHA appearances were reduced at increased temperature, as shown by ANOVA ($P < 0.05$).

### Diet impact

The LO diet induced higher disappearance of LA and ALA substrates in trout (Table 3.7), which can be logically related to their dietary large amounts. The n-6 and n-3 bioconversion pathways were positively affected by the dietary lipid source replacement. Indeed, all the n-6 fatty acid intermediates showed higher appearance levels in fish fed LO ($P < 0.01$), with the exception of 22:4n-6. Accordingly, the appearance levels of most of the n-3 fatty acid intermediates increased in fish fed LO. This was the case for all intermediates up to 22:5n-3 ($P < 0.01$), as well as for the highly valuable DHA end product ($P < 0.01$). Only the two C24 intermediates, namely 24:5n-3 and 24:6n-3, showed a decreased appearance level in fish fed LO ($P < 0.01$).

**Table 3.7.** Appearance and disappearance of fatty acids deduced by the whole body fatty acid balance method (nmol per g of fish per day) of rainbow trout reared at 15°C or 19°C on a control diet (FO) or a linseed oil diet (LO).

| | 15°C | | 19°C | | | | |
|---|---|---|---|---|---|---|---|
| Fatty acids | FO | LO | FO | LO | T | D | T × D |
| 18:2n-6 | -61.2 ± 18.4 $^{a}$ | -471.5 ± 33.2 $^{b}$ | -82.0 ± 7.0 $^{a}$ | -512.5 ± 19.1 $^{b}$ | ns | ** | ns |
| 18:3n-6 | 3.5 ± 0.3 $^{b}$ | 20.0 ± 0.7 $^{a}$ | 1.6 ± 0.2 $^{c}$ | 18.9 ± 0.6 $^{a}$ | ** | ** | ns |
| 20:2n-6 | 18.9 ± 0.9 $^{b}$ | 34.5 ± 2.3 $^{a}$ | 16.6 ± 0.5 $^{b}$ | 35.2 ± 2.2 $^{a}$ | ns | ** | ns |
| 20:3n-6 | 16.3 ± 0.5 $^{b}$ | 30.6 ± 0.8 $^{a}$ | 12.8 ± 0.3 $^{c}$ | 26.1 ± 1.2 $^{a}$ | ** | ** | ns |
| 20:4n-6 | -3.3 ± 1.1 $^{b}$ | 14.0 ± 1.2 $^{a}$ | -4.9 ± 0.7 $^{b}$ | 11.3 ± 1.0 $^{a}$ | * | ** | ns |
| 22:4n-6 | -0.8 ± 0.2 $^{a}$ | -1.2 ± 0.1 $^{ab}$ | -1.1 ± 0.1 $^{ab}$ | -1.7 ± 0.1 $^{b}$ | * | ** | ns |
| 22:5n-6 | -1.3 ± 0.8 $^{b}$ | 5.5 ± 1.0 $^{a}$ | -1.2 ± 0.4 $^{b}$ | 5.5 ± 0.5 $^{a}$ | ns | ** | ns |
| 18:3n-3 | -22.6 ± 4.0 $^{a}$ | -1785.8 ± 53.1 $^{b}$ | -14.4 ± 2.4 $^{a}$ | -1930.8 ± 41.2 $^{b}$ | * | ** | * |
| 18:4n-3 | -66.7 ± 3.0 $^{b}$ | 104.7 ± 3.1 $^{a}$ | -72.0 ± 1.6 $^{b}$ | 103.6 ± 3.1 $^{a}$ | ns | ** | ns |
| 20:3n-3 | -2.3 ± 0.9 $^{b}$ | 65.6 ± 3.7 $^{a}$ | -1.3 ± 0.9 $^{b}$ | 66.5 ± 4.0 $^{a}$ | ns | ** | ns |
| 20:4n-3 | -16.8 ± 2.1 $^{b}$ | 50.2 ± 3.6 $^{a}$ | -22.7 ± 0.4 $^{b}$ | 40.9 ± 2.4 $^{a}$ | ** | ** | ns |
| 20:5n-3 | -320.1 ± 12.8 $^{b}$ | 14.0 ± 2.1 $^{a}$ | -349.2 ± 7.5 $^{b}$ | -0.1 ± 3.2 $^{a}$ | * | ** | ns |
| 22:5n-3 | -23.6 ± 2.7 $^{b}$ | 11.7 ± 1.7 $^{a}$ | -25.1 ± 1.1 $^{b}$ | 11.7 ± 1.0 $^{a}$ | ns | ** | ns |
| 24:5n-3 | 9.9 ± 0.8 $^{a}$ | 1.2 ± 0.6 $^{b}$ | 8.9 ± 0.4 $^{a}$ | 1.1 ± 0.2 $^{b}$ | ns | ** | ns |
| 24:6n-3 | 13.8 ± 0.7 $^{a}$ | 5.0 ± 0.4 $^{b}$ | 14.1 ± 0.3 $^{a}$ | 5.7 ± 0.7 $^{b}$ | ns | ** | ns |
| 22:6n-3 | -154.3 ± 22.3 $^{b}$ | 156.1 ± 3.6 $^{a}$ | -204.1 ± 6.7 $^{b}$ | 130.1 ± 11.0 $^{a}$ | * | ** | ns |

Mean values (± SEM) within a row with no common superscript letter are significantly different (Tukey's (parametric) or Student's (nonparametric with $\alpha = 0.8$ %) *post hoc* tests). P values relative to two-way ANOVA are reported in the last three columns of the table (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; $n = 5$ except for LO at 15°C for which $n = 4$).

## Apparent *in vivo* fatty acid metabolism

### Temperature impact

The temperature increase had no impact on either the fatty acid *de novo* synthesis pathway, or on SFA and MUFA β-oxidation and elongation, or on the apparent *in vivo* Δ9 desaturation activity (Table 3.8). Moreover, in the n-6 bioconversion pathway, no significant effect of temperature was recorded on apparent *in vivo* β-oxidation, elongation and Δ5 desaturation activities involved. The apparent *in vivo* Δ6 desaturation activity was nevertheless reduced with the temperature increase ($P < 0.01$). In contrast, the apparent *in vivo* n-3 bioconversion capacity was consistently affected by temperature in fish fed LO. The n-3 apparent *in vivo* Δ5 and Δ6 desaturation activities decreased with the temperature increase ($P < 0.05$) whereas n-3 fatty acid β-oxidation increased ($P < 0.01$). The apparent *in vivo* n-3 fatty acid elongation activity was also reduced by increased temperature but not significantly ($P$ value = 0.06). Considering the sum of the fatty acid products of both n-6 and n-3 pathways, the apparent *in vivo* Δ5 and Δ6 desaturation activities were lower at the increased temperature of 19°C ($P < 0.05$). It is worth noting that the *post hoc* statistical test used for the apparent *in vivo* activity results had to be nonparametric, and was thus less powerful, which may explain why they do not corroborate the ANOVA results related to the significant effects of temperature.

### Diet impact

The LO feeding reduced the apparent *in vivo* SFA and MUFA β-oxidation ($P < 0.01$) while no impact on the *de novo* production, elongation and Δ9 desaturation was observed. On the contrary, the n-6 and n-3 bioconversion pathways were positively affected when feeding fish LO. For both pathways, the LO diet induced higher apparent *in vivo* β-oxidation, elongation, Δ5 and Δ6 desaturation enzymatic activities compared to FO at both temperatures ($P < 0.01$). Overall, considering the apparent *in vivo* fatty acid metabolism of both pathways, the elongation, Δ5 and Δ6 desaturation activities were increased in fish fed LO ($P < 0.01$).

**Table 3.8.** Fatty acid metabolism (nmol per g of fish per day), deduced by the whole body fatty acid balance method, of rainbow trout reared at 15°C or 19°C on a control diet (FO) or a linseed oil diet (LO).

| | | 15°C | | 19°C | | | | |
|---|---|---|---|---|---|---|---|---|
| | | FO | LO | FO | LO | T | D | D × T |
| **SFA and MUFA** [1] | *De novo* production | 2062 ± 89 | 1822 ± 201 | 1634 ± 91 | 1846 ± 185 | ns | ns | ns |
| | β-oxidation | 39 ± 14 [ab] | 3 ± 0 [b] | 64 ± 6 [a] | 4 ± 0 [b] | ns | ** | ns |
| | Elongation | 5114 ± 217 | 4449 ± 514 | 4048 ± 220 | 4506 ± 465 | ns | ns | ns |
| | Δ9 desaturation | 1238 ± 48 | 1082 ± 122 | 941 ± 52 | 1064 ± 109 | ns | ns | ns |
| **n-6 PUFA** [1] | β-oxidation | 33 ± 19 [b] | 368 ± 36 [a] | 58 ± 7 [b] | 417 ± 22 [a] | ns | ** | ns |
| | Elongation | 35 ± 1 [b] | 93 ± 5 [a] | 29 ± 1 [b] | 86 ± 6 [a] | ns | ** | ns |
| | Δ5 desaturation | 0 ± 0 [b] | 18 ± 2 [a] | 0 ± 0 [b] | 15 ± 1 [a] | ns | ** | ns |
| | Δ6 desaturation | 20 ± 1 [b] | 74 ± 3 [a] | 14 ± 0 [b] | 66 ± 3 [a] | ** | ** | ns |
| **n-3 PUFA** [1] | β-oxidation | 583 ± 47 [c] | 1377 ± 58 [b] | 666 ± 15 [c] | 1571 ± 36 [a] | ** | ** | ns |
| | Elongation | 26 ± 1 [b] | 640 ± 16 [a] | 23 ± 1 [b] | 541 ± 46 [a] | ns | ** | ns |
| | Δ5 desaturation | 0 ± 0 [b] | 188 ± 8 [a] | 0 ± 0 [b] | 148 ± 16 [a] | * | ** | * |
| | Δ6 desaturation | 14 ± 1 [b] | 504 ± 10 [a] | 14 ± 0 [b] | 429 ± 32 [a] | * | ** | * |
| **n-6 and n-3** [1] | Elongation | 61 ± 2 [b] | 733 ± 15 [a] | 53 ± 1 [b] | 627 ± 51 [a] | ns | ** | ns |
| **Total** | Elongation | 5175 ± 219 | 5182 ± 519 | 4101 ± 220 | 5133 ± 512 | ns | ns | ns |
| | Δ5 desaturation | 0 ± 0 [b] | 206 ± 8 [a] | 0 ± 0 [b] | 164 ± 17 [a] | * | ** | * |
| | Δ6 desaturation | 34 ± 1 [b] | 579 ± 9 [a] | 28 ± 0 [b] | 494 ± 35 [a] | * | ** | ns |

Mean values (± SEM) within a row with no common superscript letter are significantly different (Tukey's (parametric) or Student's (nonparametric with α = 0.8 %) *post hoc* tests). P values relative to two-way ANOVA are reported in the last three columns of the table (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; $n = 5$ except for LO at 15°C for which $n = 4$). [1] See Table 3.2 for abbreviations.

### 3.3.5 *fads2* and *elovl5* gene expression

The expression of *fads2* and *elovl5*, which correspond to the genes of enzymes involved in the first two steps of endogenous fatty acid bioconversion, were higher in the liver compared to the intestine (Figure 3.2). Moreover, the *elovl5* expression level was higher than that of *fads2* in the case of the liver whereas similar expression levels were observed in the intestine.

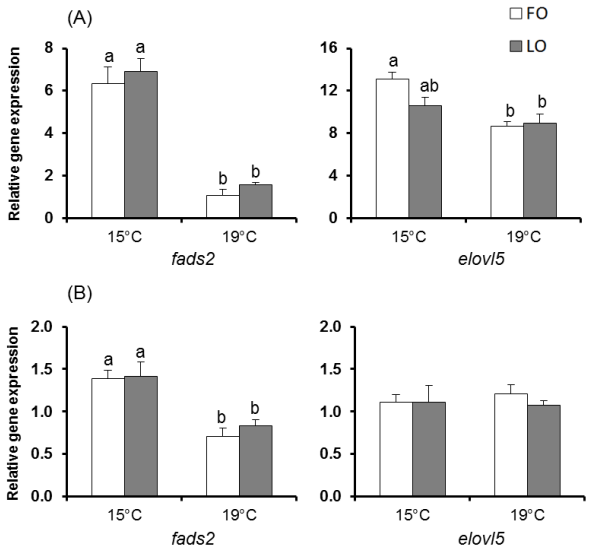


**Figure 3.2.** Effect of the dietary treatment on *fads2* and *elovl5* relative expressions in the liver (A) and intestine (B) of rainbow trout reared at two different water temperatures with a fish oil (FO) or a linseed oil (LO) diet. Results are expressed as relative mean value (± SEM) to geometric mean of *EF1α* and *β-actin* reference gene expressions. On the same graph, data with no common letter are significantly different (Tukey's (parametric) or Student's (nonparametric with α = 0.8 %) *post hoc* tests; *n* = 5 except in intestine for which *n* = 4 for LO at 19°C). *fads2*, fatty acid desaturase 2; *elovl5*, elongase 5; *EF1α*, elongation factor 1α.

**Temperature impact**

Irrespective of diet, *fads2* expression was reduced by the increased temperature of 19°C, both in the liver and intestine. In contrast, *elovl5* expression was negatively impacted by the temperature increase, but only in liver in fish fed FO.

**Diet impact**

The dietary treatment did not affect the expression of *fads2* or *elovl5* in either tissue ($P > 0.05$).

### 3.3.6 Δ6 Desaturase enzymatic activity

**Temperature impact**

The temperature increase induced a six-fold reduction of the Δ6 desaturase enzymatic activity, measured as the rate of desaturation of [1-$1^4$C]18:3n-3 to [1-$^{14}$C]18:4n-3 by intestinal microsomes (Figure 3.3). The difference was however significant only for fish fed LO.

**Diet impact**

The Δ6 desaturase enzymatic activity was not affected by the dietary treatment in intestine, irrespective of temperature (Figure 3.3). Accordingly, no dietary impact was observed on liver microsomes of fish held at 19°C (3.41 ± 0.8 and 3.93 ± 0.53 pmol × $h^{-1}$ × mg $protein^{-1}$ for fish fed FO and LO, respectively). Interestingly, the Δ6 desaturase enzymatic activity was more than 20-fold higher in liver microsomes than in intestinal microsomes of fish held at 19°C. Unfortunately, due to technical problems with some samples, no results are available for the liver microsomes of fish reared at 15°C.

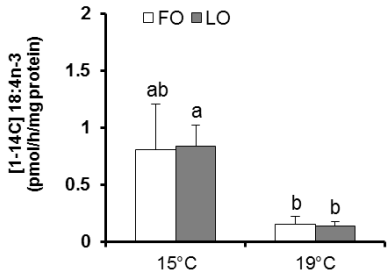


**Figure 3.3.** Δ6 Desaturase enzymatic activity (pmol of [1-14C]18:4n-3 by hour and mg of protein) of intestinal microsomes of rainbow trout reared at two different water temperatures on a control diet (FO) or a linseed oil diet (LO). Mean values (± SEM) with no common letter are significantly different (Tukey's *post hoc* test on log transformed values; $n = 5$).

## 3.4 Discussion

The present study aimed to evaluate the impact of a 4°C increase in water temperature on the fatty acid bioconversion capacity of rainbow trout fed a LO diet. It had already been reported that this species possesses a good fatty acid bioconversion capacity when fed with plant-derived oils [24, 25, 52-54]. However, less is known about this metabolic capacity in warmer water.

Regarding fish growth performance, the present data indicated that replacing fish oil with linseed oil had nutritional troubles with increased water temperature beyond the limit of the optimal temperature range of rainbow trout (15°C - 16°C). In accordance to what was observed in the present study, several earlier studies also described that, in salmonids, increased water temperature can negatively affect fish growth by increasing feed intake [15, 34] but decreasing feed efficiency [55] and hepatosomatic index [34, 55, 56]. Moreover, it has been reported that rainbow trout energy requirements increase, almost linearly, with water temperature [6, 20] and, therefore, induce higher feed intake and lower dietary protein and lipid conversion and retention, as highlighted in the present study. In contrast, the results of published studies are partly contradictory to ours. For example, while feeding adult Atlantic salmon a diet formulated with a blend of fish and rapeseed oils for 56 days, Hevrøy *et al.* [55] found, as here, negative effects of elevated temperature from 14°C to 19°C on growth, feed utilisation, NRE, LRE and hepatosomatic index but feed intake was also negatively impacted. Considering the effect of lipid source replacement on fish growth, the total or substantial replacement of fish oil by linseed oil had already been shown to have no detrimental effect on salmonid growth when fish were held at their optimal growth temperature [18, 23-26, 57]. For fish held at 19°C in the present study, the feed intake and overall feed efficiency were unaffected by dietary treatment but, inconsistently, reduced conversion and retention efficiencies of the dietary lipids (LER and LRE, respectively) were observed. This might be due to the length of the feeding trial, which could have been too short to affect feed efficiency but long enough to allow differences of the LER and LRE coefficients. The reduced LER and LRE observed when the dietary change was imposed at 19°C could indicate a higher fish energy demand in that condition and explain the reduced DGC observed.

In the present study, significantly higher ALA was recorded with the temperature increase in the whole body of fish fed LO. Conversely, the

temperature increase slightly reduced the fish n-3 LC-PUFA content. A similar temperature effect on ALA content but an absence of effect on n-3 LC-PUFA content have been reported previously in the whole body of Atlantic salmon fed diets varying in the ARA/EPA ratio from 160 g to around 250 g [34]. Interestingly, the temperature increase slightly increased ALA but highly reduced EPA and DHA in European sea bass (*Dicentrarchus labrax*) fed a rapeseed oil diet [38]. Regarding the n-6 PUFA profile, the temperature increase induced an increase of LA and a slight decrease of n-6 LC-PUFA content. However, no temperature effect was observed on ARA, which is in accordance with data previously reported in European sea bass reared at 22°C or 29°C and fed a rapeseed oil diet [38]. In contrast, increased ARA was reported in Atlantic salmon when water temperature was increased from 10°C to 20°C [34]. As regards the diet impact on fish lipid composition, increased fatty acid bioconversion of LA and ALA in fish fed LO was associated with increased levels of their corresponding products until 20:3n-6 and 20:4n-3, respectively. In contrast, the final n-3 bioconversion products, EPA and DHA, decreased when feeding fish with LO, leading to a reduced n-3 LC-PUFA content with LO. As observed in the present paper and reported by numerous previous studies [23-26, 30, 57], fish fatty acid composition after a feeding trial reflects that of the experimental diet administered. Concerning n-3 LC-PUFA, Tocher *et al.* [58] reported that the fatty acid bioconversion capacity of Atlantic salmon fed a linseed oil diet was increased until 20:4n-3 but ineffective in maintaining similar EPA and DHA contents than fish fed a fish oil diet, despite high dietary ALA, which is consistent with the present data. In order to counteract the EPA and DHA decrease by feeding fish with plant-derived oils, finishing diets, fed at the end of the fish grow-out period and formulated with fish oil, have been investigated to restore the n-3 LC-PUFA content in fish fillet and have shown positive results [18, 23, 24]. Moreover, a study demonstrated that water temperature had no influence on this restoration capacity in rainbow trout held at 15°C or 20°C [59].

The whole body fatty acid balance method previously developed by Turchini *et al.* [40, 41, 46] highlighted firstly a higher disappearance of ALA with the temperature increase. However, this higher disappearance was not correlated with higher appearance of n-3 fatty acid bioconversion products. Regarding the apparent *in vivo* enzymatic activities, the elongases apparent *in vivo* activity was not impacted by temperature considering both the n-6 and n-3 pathways. In contrast, the apparent *in vivo* activities of the Δ5 and Δ6

desaturases were slightly reduced at the increased temperature of 19°C. A recently published study [33] also investigated the impact of a temperature increase on fish lipid metabolism with the use of the whole body fatty acid balance method. This study investigated Atlantic salmon raised at the increased temperature of 20°C, in comparison with 10°C, and fed diets varying in ARA/EPA ratio. Consistent with data in the present study, the authors reported a reduced apparent *in vivo* Δ6 desaturation activity and no effect on the Δ9 desaturation activity. In contrast however, no effect on Δ5 desaturation was observed. Regarding the elongation activities, contrasting data were obtained since the apparent *in vivo* activities of elovl5 and elovl2 were not modified and reduced, respectively [33]. A positive effect of linseed oil diets on the rainbow trout fatty acid bioconversion capacity when fish were held at their optimal growth temperature has already been reported [24, 25]. The present results are in accordance with these studies regarding *e.g.* the higher apparent *in vivo* elongase, Δ5 and Δ6 desaturase activities for fish fed LO.

The present results indicated reduced *fads2* expression in liver and intestine with the temperature increase. Moreover, *elovl5* expression was also reduced in liver of fish fed FO. These results are consistent with those of Norambuena *et al.* [33] on Atlantic salmon held at 10°C or 20°C, which showed reduced hepatic elongase (*elovl2*) and *fads2* gene expressions with increased temperature. In contrast, similar Δ6 desaturase mRNA levels were measured at 16°C and 22°C in European sea bass larvae fed a n-3 LC-PUFA deprived diet [39]. In contrast to the impact of temperature, the diet had no effect on *fads2* and *elovl5* expressions. These results are in contradiction with previous studies on salmonids that reported increased desaturase and elongase gene expressions in fish fed plant-derived oils due to the reduced dietary levels of n-3 LC-PUFA, that suppress the expression of these genes [31, 53, 54, 60, 61]. Moreover, several studies already reported that the replacement of fish oil by plant-derived oils increased Δ6 desaturase gene mRNA levels in freshwater fish (see review [53]).

Consistent with the results obtained on the effect of temperature on *fads2* expression, microsomal Δ6 desaturase activity measured in the intestine of fish fed LO was reduced with the temperature increase. An increase in water temperature had been previously reported to reduce desaturase activity in freshwater fish [35, 62, 63]. This effect was in line with the importance of that enzyme for increased cell membrane fluidity associated with cold

acclimation. In accordance, Hagar and Hazel [62] observed increased Δ6 desaturase activity in liver microsomes of rainbow trout when acclimating from 20°C to 5°C. De Torrengo and Brenner [63] also reported that Δ6 desaturase activity was increased in liver microsomes of catfish (*Pimelodus maculatus*) in case of cold acclimation. Furthermore, in rainbow trout, a drop in the desaturation enzymatic activity in hepatocytes and enterocytes has been observed in fish raised at 15°C as compared to 7°C and 11°C [35]. In contrast with the temperature impact, no effect of dietary treatment on microsomal Δ6 desaturase enzymatic activity was observed. In accordance with the present study, no difference in Δ6 desaturase activity was observed in liver and intestinal microsomes of Eurasian perch (*Perca fluviatilis*) fed fish oil or linseed oil diets [49]. However, our results are conflicting with most of the previous studies reporting that Δ6 desaturase activity is under nutritional regulation in fish. Indeed, increased Δ6 desaturase activity was observed in liver microsomes of rainbow trout fed an olive oil diet low in n-3 LC-PUFA, compared to fish fed a fish oil diet [52]. Moreover, numerous studies on isolated hepatocytes showed that plant-derived oil diets consistently induced higher Δ6 desaturase activity in freshwater fish (see review [53]). The discrepancy between these results could be explained by the different methods used (microsome assay *vs* isolated hepatocyte assay). Our results lead us to conclude that, when measured in microsomes, dietary influences on Δ6 desaturase activity were not apparent. It could be interesting to repeat the analysis on isolated hepatocytes in order to study also the impact of cellular environment on Δ6 desaturase activity.

Overall, the present results indicate a negative impact of a temperature increase close to the upper limit of the species temperature tolerance range on the feed efficiency and the fatty acid metabolism of rainbow trout fed a linseed oil diet. Previous studies had reported that rainbow trout possesses a good fatty acid bioconversion capacity at its optimal growth temperature [29, 30, 53] and that this capacity was increased in case of cold acclimation, altering cell membrane fluidity [62, 64]. In warm acclimation, the increased fluidity is unnecessary and the bioconversion capacity not stimulated. Rather than no detrimental effect, increased temperature induced a pronounced negative impact on fish metabolism with decreased desaturase and elongase gene expression and reduced Δ6 desaturase enzymatic activity. To a lesser extent, a negative effect of temperature was also indicated by slightly reduced apparent *in vivo* enzymatic activity of Δ5 and Δ6 desaturases. This less obvious effect emphasised the basal strong capacity of rainbow trout to

endogenously produce n-3 LC-PUFA from the n-3 precursor ALA and a particular resistance of this species to external influences, such as water temperature. Despite the detrimental temperature effects on fatty acid metabolism, the whole fish n-3 LC-PUFA content was only slightly affected by temperature. However, a longer trial under similar experimental conditions with fish reaching a marketable size should nevertheless be performed to support the present results and lead to a final conclusion on the water temperature impact, in a human nutrition perspective. In contrast to the temperature effect, dietary lipid source replacement greatly reduced the n-3 LC-PUFA content of rainbow trout. Despite the positive response of fish to the ALA-rich linseed oil diet in terms of increased apparent *in vivo* desaturase and elongase activities, the EPA and DHA contents in fish fed LO did not match those in fish fed FO. More research is thus required on the replacement of fish oil by readily available, economically and environmentally sustainable lipid source alternatives in aquaculture feeds. In this context, studies on transgenic plants rich in EPA and DHA [65], finishing diets [18, 23, 24] or modulators [66, 67] added to feed to improve fish lipid bioconversion capacity could be potential options.

## 3.5 Supporting Information

**S3.1 Table.** Apparent digestibility coefficients (%) of fatty acids for the control diet (FO) and the linseed oil diet (LO) used to determine appearance and disappearance of fatty acids (Table 3.7) and apparent *in vivo* fatty acid metabolism (Table 3.8).

| | Diet | |
|---|---|---|
| Fatty acid | FO | LO |
| 12:0 | 84 | 89 |
| 14:0 | 89 | 75 |
| 16:0 | 85 | 90 |
| 18:0 | 83 | 92 |
| 20:0 | 71 | 83 |
| 22:0 | 100 | 68 |
| 24:0 | 87 | 83 |
| 14:1n-5 | 91 | 100 |
| 16:1n-7 | 91 | 50 |
| 18:1n-7 | 89 | 90 |
| 18:1n-9 | 90 | 95 |
| 20:1n-9 | 90 | 100 |
| 22:1n-9 | 100 | 100 |
| 24:1n-9 | 82 | 100 |
| 20:1n-11 | 100 | 100 |
| 22:1n-11 | 100 | 100 |
| 22:1n-13 | 100 | 100 |
| 18:2n-6 | 91 | 96 |
| 20:2n-6 | 87 | 62 |
| 22:2n-6 | 100 | 100 |
| 18:3n-6 | 97 | 99 |
| 20:3n-6 | 83 | 100 |
| 20:4n-6 | 93 | 83 |
| 22:4n-6 | 100 | 100 |
| 24:4n-6 | 100 | 100 |
| 24:5n-6 | 100 | 100 |
| 22:5n-6 | 94 | 100 |
| 18:3n-3 | 87 | 98 |
| 20:3n-3 | 99 | 85 |
| 22:3n-3 | 100 | 100 |
| 18:4n-3 | 96 | 100 |
| 20:4n-3 | 95 | 100 |
| 20:5n-3 | 96 | 89 |
| 22:5n-3 | 96 | 100 |
| 24:5n-3 | 94 | 98 |
| 24:6n-3 | 100 | 100 |
| 22:6n-3 | 95 | 85 |

## 3.6 References


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## 3.7 Additional results

In addition to the fatty acid composition of whole fish, the fatty acid profile of fish fillet and adipose tissue were analysed. The results are presented and discussed in the following section.

### 3.7.1 Experimental procedures

At the end of the feeding trial as described in Section 3.2.3, the fillet and adipose tissue were collected at the same time as the liver and intestine from seven fish. The fresh tissues were afterwards frozen in liquid nitrogen and stored at – 20°C until analysis.

The fillet and adipose tissue fatty acid profiles were analysed as described in Section 3.2.6 for the whole fish and diets with the exception of the lipid extraction method. The fillet and adipose tissue lipid extractions were performed according to a method adapted from Bligh and Dyer [1] using chloroform/methanol/water (2:2:1.8, *v:v:v*) (VWR chemicals, Radnor, PA, USA) with 100 mg of homogenised tissue. Each tissue lipid extract was then separated into the free fatty acid (FFA), phospholipid (PL) and neutral lipid (NL) fractions by solid phase extraction, as previously described [2]. Briefly, lipids were loaded on a Bond Elut-$NH_2$ column (200 mg, Varian, Middelburg, The Netherlands). The NL fraction was eluted with chloroform/2-propanol (2:1, *v:v*) (VWR chemicals), the FFA fraction by diethyl ether/acetic acid (98:2, *v:v*) (VWR chemicals) and the PL fraction by methanol (VWR chemicals). Tridecanoic acid, 1,2-dipentadecanoyl-sn-glycero-3-phosphatidylcholine and triheptadecanoin (Larodan, Solna, Sweden) were used as internal standards for lipid quantification in the FFA, PL and NL fractions, respectively. Only the fatty acid profiles of fillet NL and PL fractions and the NL fraction of adipose tissue are reported. Indeed, the FFA fractions remained marginal for both tissues, whereas the adipose tissue PL fraction could not deliver an acute fatty acid profile because of the limitation of the extraction method. The final results are expressed in mg/g of dry tissue. The DM was analysed as described in Section 3.2.4.

The statistical analysis was performed as described in Section 3.2.9.

### 3.7.2 Results

The fatty acid composition of the fillet NL and PL fractions are reported in Table 3.9 and the fatty acid composition of the adipose tissue NL fraction in Table 3.10.

**Temperature impact**

Overall, the temperature increase had no major impact on the fatty acid composition of the NL and PL fractions of rainbow trout fillet (Table 3.9), with the exception of the n-3/n-6 ratio, which slightly increased with increased temperature in NL ($P < 0.05$), and the MUFA content which slightly decreased in PL ($P < 0.01$). Regarding the adipose tissue NL fatty acid composition (Table 3.10) of fish fed LO, the amounts of C18 n-6 and n-3 PUFA increased with the temperature increase, especially the LA and ALA contents, respectively ($P < 0.01$). Still for fish fed LO, the adipose tissue n-6 and n-3 LC-PUFA contents also increased with the temperature increase ($P < 0.01$). The higher n-6 LC-PUFA content was explained by the increase of 20:2n-6 and 22:5n-6, whereas the higher n-3 LC-PUFA content was explained by an increase of all the n-3 bioconverted fatty acids, namely 20:4n-3, EPA, 22:5n-3, 24:5n-3, 24:6n-3 and DHA. It is however worth noting that these increases in n-6 and n-3 fatty acids were supported by the ANOVA but not corroborated by the *post hoc* results.

**Diet impact**

Regarding both NL and PL fractions of the fillet, C18 n-6 and n-3 PUFA contents increased in fillet of fish fed LO (Table 3.9). In fillet of fish held at 19°C, the LA content increased in both lipid fractions with dietary replacement and higher 18:3n-6 content was only observed in NL. In contrast, higher contents of ALA and 18:4n-3 were recorded in both lipid fractions of fillet at both temperatures. The n-6 LC-PUFA content was not impacted by dietary treatment in fillet. The opposite was however observed for the n-3 LC-PUFA content. Indeed, despite an increase of 20:3n-3 and 20:4n-3, the reduced amounts of EPA and DHA led to decreased the n-3 LC-PUFA content in fillet NL and PL of fish fed LO. Regarding the adipose tissue NL, C18 n-6 and n-3 PUFA increased in fish fed LO, due to increased levels of LA and ALA, respectively, but also to increased desaturated product of ALA, namely 18:4n-3 (Table 3.10). Considering the ANOVA results, the n-6 LC-PUFA content slightly increased when feeding fish with

LO ($P < 0.05$). In contrast, the n-3 LC-PUFA content in adipose tissue decreased in fish fed LO ($P < 0.01$), due to moderate increases in 20:3n-3 and 20:4n-3 ($P < 0.01$) as compared to large reductions in EPA, 22:5n-3, 24:5n-3, 24:6n-3 and DHA ($P < 0.01$). Consequently, the n-3/n-6 ratio increased with dietary replacement in NL of both tissues whereas the opposite was observed for the n-3 LC-PUFA / n-6 LC-PUFA ratio.

**Temperature × Diet impact**

A temperature × diet interaction effect was observed for the LA content of PL of fish fillet ($P < 0.05$) due to the reduction in this content for fish fed FO with increased temperature whereas no effect was observed for fish fed LO. A second interaction effect was observed on the C18 n-3 PUFA content and ALA in NL of adipose tissue ($P < 0.05$), as no effect was observed for fish fed FO whereas a significant increase with the temperature increase was reported for fish fed LO.

**Table 3.9.** Fatty acid profile (mg/g of dry matter) of neutral lipid and phospholipid fractions of the fillet of rainbow trout reared at 15°C or 19°C on a control diet (FO) or a linseed oil diet (LO) [1].

| | Neutral lipids | | | | | | |
|---|---|---|---|---|---|---|---|
| | 15°C | | 19°C | | | | |
| Fatty acids | FO | LO | FO | LO | T | D | T × D |
| 18:2n-6 | 5.21 ± 0.51 [ab] | 6.46 ± 0.71 [a] | 4.12 ± 0.40 [b] | 6.58 ± 0.52 [a] | ns | ** | ns |
| 18:3n-6 | 0.12 ± 0.02 [ab] | 0.15 ± 0.01 [a] | 0.08 ± 0.01 [b] | 0.16 ± 0.01 [a] | ns | ** | ns |
| 20:2n-6 | 0.37 ± 0.03 | 0.45 ± 0.06 | 0.41 ± 0.05 | 0.42 ± 0.03 | ns | ns | ns |
| 20:3n-6 | 0.22 ± 0.02 [ab] | 0.28 ± 0.03 [a] | 0.16 ± 0.02 [b] | 0.28 ± 0.02 [a] | ns | ** | ns |
| 20:4n-6 | 0.28 ± 0.03 [a] | 0.16 ± 0.02 [b] | 0.21 ± 0.01 [ab] | 0.16 ± 0.03 [b] | ns | ** | ns |
| 22:4n-6 | 0.09 ± 0.04 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.03 ± 0.03 | ns | ns | * |
| 22:5n-6 | 0.05 ± 0.03 | 0.03 ± 0.02 | 0.06 ± 0.02 | 0.02 ± 0.02 | ns | ns | ns |
| 18:3n-3 | 0.55 ± 0.05 [b] | 8.04 ± 0.77 [a] | 0.57 ± 0.07 [b] | 8.32 ± 0.62 [a] | ns | ** | ns |
| 18:4n-3 | 0.47 ± 0.07 [b] | 0.81 ± 0.08 [a] | 0.37 ± 0.04 [b] | 0.84 ± 0.07 [a] | ns | ** | ns |
| 20:3n-3 | 0.13 ± 0.06 [b] | 0.64 ± 0.14 [a] | 0.18 ± 0.02 [b] | 0.55 ± 0.11 [a] | ns | ** | ns |
| 20:4n-3 | 0.33 ± 0.02 [ab] | 0.45 ± 0.06 [a] | 0.25 ± 0.03 [b] | 0.51 ± 0.08 [a] | ns | ** | ns |
| 20:5n-3 | 1.49 ± 0.25 [a] | 0.64 ± 0.06 [b] | 1.31 ± 0.13 [a] | 0.68 ± 0.08 [b] | ns | ** | ns |
| 22:5n-3 | 0.61 ± 0.07 [a] | 0.27 ± 0.02 [c] | 0.48 ± 0.06 [ab] | 0.31 ± 0.03 [bc] | ns | ** | ns |
| 24:5n-3 | 0.17 ± 0.02 [a] | 0.05 ± 0.01 [b] | 0.12 ± 0.01 [a] | 0.05 ± 0.01 [b] | ns | ** | ns |
| 24:6n-3 | 0.19 ± 0.02 | 0.07 ± 0.02 | 0.19 ± 0.02 | 0.06 ± 0.02 | ns | ** | ns |
| 22:6n-3 | 5.11 ± 0.47 [a] | 2.29 ± 0.23 [b] | 4.35 ± 0.34 [a] | 2.56 ± 0.26 [b] | ns | ** | ns |

**Table 3.9** - Continued

| | Neutral lipids | | | | | | |
|---|---|---|---|---|---|---|---|
| | 15°C | | 19°C | | | | |
| Fatty acids | FO | LO | FO | LO | T | D | T × D |
| Σ SFA | 21.45 ± 2.36 [a] | 14.86 ± 1.86 [ab] | 17.83 ± 1.53 [ab] | 13.89 ± 1.31 [b] | ns | ** | ns |
| Σ MUFA | 33.30 ± 3.39 [a] | 21.84 ± 2.64 [b] | 26.14 ± 2.79 [ab] | 19.68 ± 1.65 [b] | ns | ** | ns |
| Σ C18 n-6 PUFA | 5.33 ± 0.52 [ab] | 6.61 ± 0.73 [a] | 4.20 ± 0.41 [b] | 6.74 ± 0.53 [a] | ns | ** | ns |
| Σ n-6 LC-PUFA | 1.01 ± 0.12 | 0.92 ± 0.10 | 0.83 ± 0.07 | 0.91 ± 0.06 | ns | ns | ns |
| Σ C18 n-3 PUFA | 1.02 ± 0.11 [b] | 8.85 ± 0.84 [a] | 0.94 ± 0.11 [b] | 9.17 ± 0.67 [a] | ns | ** | ns |
| Σ n-3 LC-PUFA | 8.03 ± 0.89 [a] | 4.42 ± 0.39 [c] | 6.89 ± 0.59 [ab] | 4.73 ± 0.33 [bc] | ns | ** | ns |
| n-3/n-6 | 1.42 ± 0.04 [b] | 1.77 ± 0.04 [a] | 1.56 ± 0.03 [b] | 1.82 ± 0.04 [a] | * | ** | ns |
| n-3 LC-PUFA /n-6 LC-PUFA | 8.10 ± 0.64 [a] | 4.87 ± 0.19 [b] | 8.32 ± 0.53 [a] | 5.21 ± 0.35 [b] | ns | ** | ns |
| Total | 70.35 ± 7.33 | 57.62 ± 6.48 | 57.26 ± 5.47 | 55.17 ± 4.37 | ns | ns | ns |

**Table 3.9** - Continued

| | Phospholipids | | | | | | |
|---|---|---|---|---|---|---|---|
| | 15°C | | 19°C | | | | |
| Fatty acids | FO | LO | FO | LO | T | D | T × D |
| 18:2n-6 | 0.72 ± 0.02 [b] | 1.22 ± 0.04 [a] | 0.61 ± 0.01 [c] | 1.24 ± 0.04 [a] | ns | ** | * |
| 18:3n-6 | 0.02 ± 0.01 | 0.03 ± 0.02 | 0.01 ± 0.01 | 0.01 ± 0.01 | ns | ns | ns |
| 20:2n-6 | 0.14 ± 0.08 | 0.14 ± 0.01 | 0.06 ± 0.03 | 0.19 ± 0.09 | ns | ns | ns |
| 20:3n-6 | 0.15 ± 0.02 [ab] | 0.24 ± 0.02 [a] | 0.09 ± 0.02 [b] | 0.27 ± 0.02 [a] | ns | ** | ns |
| 20:4n-6 | 0.19 ± 0.01 | 0.16 ± 0.01 | 0.13 ± 0.03 | 0.14 ± 0.04 | ns | ns | ns |
| 22:4n-6 | 0.08 ± 0.05 | 0.00 ± 0.00 | 0.01 ± 0.00 | 0.04 ± 0.04 | ns | ns | ns |
| 22:5n-6 | 0.04 ± 0.03 | 0.03 ± 0.02 | 0.04 ± 0.02 | 0.01 ± 0.01 | ns | ns | ns |
| 18:3n-3 | 0.03 ± 0.02 [b] | 1.28 ± 0.06 [a] | 0.03 ± 0.02 [b] | 1.43 ± 0.05 [a] | ns | ** | ns |
| 18:4n-3 | 0.06 ± 0.03 [b] | 0.46 ± 0.01 [a] | 0.03 ± 0.02 [b] | 0.37 ± 0.03 [a] | ns | ** | ns |
| 20:3n-3 | 0.01 ± 0.00 [b] | 0.09 ± 0.01 [a] | 0.00 ± 0.00 [b] | 0.12 ± 0.01 [a] | ns | ** | ns |
| 20:4n-3 | 0.12 ± 0.01 [b] | 0.31 ± 0.03 [a] | 0.12 ± 0.01 [b] | 0.37 ± 0.08 [a] | ns | ** | ns |
| 20:5n-3 | 0.86 ± 0.05 [a] | 0.66 ± 0.04 [b] | 0.91 ± 0.02 [a] | 0.66 ± 0.03 [b] | ns | ** | ns |
| 22:5n-3 | 0.23 ± 0.01 | 0.20 ± 0.01 | 0.23 ± 0.01 | 0.22 ± 0.01 | ns | ns | ns |
| 24:5n-3 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | ns | ns | ns |
| 24:6n-3 | 0.02 ± 0.02 | 0.02 ± 0.01 | 0.02 ± 0.01 | 0.01 ± 0.01 | ns | ns | ns |
| 22:6n-3 | 5.31 ± 0.14 [a] | 3.76 ± 0.09 [b] | 5.13 ± 0.05 [a] | 3.93 ± 0.12 [b] | ns | ** | ns |

**Table 3.9** - Continued

| | Phospholipids | | | | | | |
|---|---|---|---|---|---|---|---|
| | 15°C | | 19°C | | | | |
| Fatty acids | FO | LO | FO | LO | T | D | T × D |
| Σ SFA | 5.29 ± 0.51 | 4.83 ± 0.39 | 4.97 ± 0.33 | 5.36 ± 0.49 | ns | ns | ns |
| Σ MUFA | 3.82 ± 0.11 [a] | 3.26 ± 0.09 [b] | 3.28 ± 0.07 [b] | 3.03 ± 0.06 [b] | ** | ** | ns |
| Σ C18 n-6 PUFA | 0.73 ± 0.02 [b] | 1.25 ± 0.05 [a] | 0.63 ± 0.02 [c] | 1.25 ± 0.04 [a] | ns | ** | ns |
| Σ n-6 LC-PUFA | 0.61 ± 0.14 | 0.57 ± 0.02 | 0.32 ± 0.09 | 0.65 ± 0.12 | ns | ns | ns |
| Σ C18 n-3 PUFA | 0.09 ± 0.04 [b] | 1.74 ± 0.07 [a] | 0.06 ± 0.04 [b] | 1.80 ± 0.05 [a] | ns | ** | ns |
| Σ n-3 LC-PUFA | 6.55 ± 0.18 [a] | 5.04 ± 0.13 [b] | 6.40 ± 0.04 [a] | 5.30 ± 0.13 [b] | ns | ** | ns |
| n-3/n-6 | 5.17 ± 0.54 [ab] | 3.73 ± 0.13 [b] | 7.13 ± 0.82 [a] | 3.76 ± 0.15 [b] | ns | ** | ns |
| n-3 LC-PUFA /n-6 LC-PUFA | 12.96 ± 2.54 | 8.88 ± 0.46 | 16.51 ± 1.92 | 9.12 ± 1.37 | ns | ns | ns |
| Total | 17.11 ± 0.70 | 16.68 ± 0.31 | 15.68 ± 0.25 | 17.39 ± 0.66 | ns | ns | ns |

Mean values (± SEM) within a row with no common superscript letter are significantly different (Tukey's (parametric) or Student's (nonparametric with α = 0.8 %) *post hoc* tests on square root transformed values by fraction). P values relative to two-way ANOVA are reported in the last three columns by fraction (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; $n = 5$).[1] See Table 3.2 for abbreviations.

**Table 3.10.** Fatty acid profile (mg/g of dry matter) of the adipose tissue neutral lipid fraction of rainbow trout reared at 15°C or 19°C on a control diet (FO) or a linseed oil diet (LO) [1].

| | 15°C | | 19°C | | | | |
|---|---|---|---|---|---|---|---|
| Fatty acids | FO | LO | FO | LO | T | D | T × D |
| 18:2n-6 | 40.74 ± 2.17 $^{d}$ | 64.65 ± 2.13 $^{b}$ | 49.51 ± 1.91 $^{c}$ | 82.28 ± 3.59 $^{a}$ | ** | ** | ns |
| 18:3n-6 | 0.87 ± 0.08 | 1.49 ± 0.21 | 0.86 ± 0.05 | 1.79 ± 0.27 | ns | ** | ns |
| 20:2n-6 | 2.52 ± 0.11 $^{b}$ | 3.45 ± 0.20 $^{ab}$ | 3.00 ± 0.33 $^{b}$ | 4.49 ± 0.40 $^{a}$ | * | ** | ns |
| 20:3n-6 | 1.54 ± 0.15 $^{b}$ | 2.15 ± 0.05 $^{a}$ | 1.54 ± 0.12 $^{b}$ | 2.65 ± 0.21 $^{a}$ | ns | ** | ns |
| 20:4n-6 | 1.84 ± 0.22 $^{ab}$ | 1.36 ± 0.13 $^{b}$ | 2.09 ± 0.13 $^{a}$ | 1.39 ± 0.12 $^{b}$ | ns | ** | ns |
| 22:4n-6 | 0.35 ± 0.12 | 0.16 ± 0.01 | 0.29 ± 0.02 | 0.19 ± 0.02 | ns | * | ns |
| 22:5n-6 | 0.71 ± 0.06 $^{ab}$ | 0.47 ± 0.08 $^{b}$ | 0.91 ± 0.06 $^{a}$ | 0.83 ± 0.11 $^{a}$ | ** | * | ns |
| 18:3n-3 | 3.30 ± 0.50 $^{c}$ | 73.69 ± 2.72 $^{b}$ | 5.36 ± 0.73 $^{c}$ | 95.88 ± 3.91 $^{a}$ | ** | ** | * |
| 18:4n-3 | 3.60 ± 0.31 $^{b}$ | 7.31 ± 0.22 $^{a}$ | 4.44 ± 0.34 $^{b}$ | 9.50 ± 1.22 $^{a}$ | * | ** | ns |
| 20:3n-3 | 0.30 ± 0.18 $^{b}$ | 3.55 ± 0.27 $^{a}$ | 0.42 ± 0.26 $^{b}$ | 4.36 ± 0.34 $^{a}$ | ns | ** | ns |
| 20:4n-3 | 2.62 ± 0.16 $^{c}$ | 4.08 ± 0.22 $^{ab}$ | 3.10 ± 0.21 $^{bc}$ | 6.43 ± 0.64 $^{a}$ | ** | ** | * |
| 20:5n-3 | 11.31 ± 1.71 $^{ab}$ | 7.01 ± 0.48 $^{b}$ | 14.41 ± 1.29 $^{a}$ | 8.70 ± 0.54 $^{b}$ | * | ** | ns |
| 22:5n-3 | 4.58 ± 0.41 $^{ab}$ | 2.97 ± 0.10 $^{c}$ | 5.87 ± 0.49 $^{a}$ | 4.05 ± 0.25 $^{bc}$ | ** | ** | ns |
| 24:5n-3 | 1.30 ± 0.09 $^{a}$ | 0.69 ± 0.03 $^{b}$ | 1.50 ± 0.08 $^{a}$ | 0.89 ± 0.05 $^{b}$ | ** | ** | ns |
| 24:6n-3 | 1.60 ± 0.18 $^{ab}$ | 0.85 ± 0.08 $^{c}$ | 2.00 ± 0.12 $^{a}$ | 1.16 ± 0.09 $^{bc}$ | ** | ** | ns |
| 22:6n-3 | 33.89 ± 2.78 $^{ab}$ | 19.90 ± 1.22 $^{c}$ | 40.35 ± 3.07 $^{a}$ | 25.55 ± 1.43 $^{bc}$ | * | ** | ns |

**Table 3.10** - Continued

| | 15°C | | 19°C | | | | |
|---|---|---|---|---|---|---|---|
| Fatty acids | FO | LO | FO | LO | T | D | T × D |
| Σ SFA | 171.77 ± 7.67 [ab] | 146.64 ± 4.70 [c] | 185.41 ± 6.57 [a] | 157.45 ± 3.25 [bc] | * | ** | ns |
| Σ MUFA | 295.95 ± 16.48 [a] | 227.67 ± 7.77 [b] | 316.00 ± 8.57 [a] | 244.12 ± 5.94 [b] | ns | ** | ns |
| Σ C18 n-6 PUFA | 41.61 ± 2.23 [c] | 66.14 ± 2.30 [b] | 50.37 ± 1.95 [c] | 84.07 ± 3.62 [a] | ** | ** | ns |
| Σ n-6 LC-PUFA | 6.97 ± 0.41 [b] | 7.59 ± 0.35 [b] | 7.83 ± 0.55 [ab] | 9.54 ± 0.45 [a] | ** | * | ns |
| Σ C18 n-3 PUFA | 6.91 ± 0.72 [c] | 81.00 ± 2.92 [b] | 9.80 ± 1.00 [c] | 105.38 ± 4.74 [a] | ** | ** | * |
| Σ n-3 LC-PUFA | 55.61 ± 4.97 [ab] | 39.04 ± 1.94 [c] | 67.65 ± 5.27 [a] | 51.14 ± 1.97 [bc] | ** | ** | ns |
| n-3/n-6 | 1.28 ± 0.05 [b] | 1.63 ± 0.03 [a] | 1.32 ± 0.06 [b] | 1.67 ± 0.00 [a] | ns | ** | ns |
| n-3 LC-PUFA /n-6 LC-PUFA | 7.95 ± 0.42 [a] | 5.16 ± 0.25 [b] | 8.64 ± 0.34 [a] | 5.38 ± 0.20 [b] | ns | ** | ns |
| Total | 580.81 ± 31.24 | 568.47 ± 18.64 | 640.91 ± 23.24 | 652.83 ± 19.08 | ** | ns | ns |

Mean values (± SEM) within a row with no common superscript letter are significantly different (Tukey's (parametric) or Student's (nonparametric with α = 0.8 %) *post hoc* tests on square root transformed values). P values relative to two-way ANOVA are reported in the last three columns of the table (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; $n = 5$).

[1] See Table 3.2 for abbreviations.

### 3.7.3 Discussion

In the present study, the n-3 PUFA composition of fillet was unaffected by the temperature increase. Unaltered fillet n-3 LC-PUFA content could be seen as positive considering human nutrition since fish are the major source of n-3 LC-PUFA. However, the present study was carried out with fish with a final weight of around 50 g and conclusions on the temperature impact on fillet in a human nutrition aspect have to be taken with caution. A longer trial under similar experimental conditions should be performed throughout the grow-out period until a marketable final fish size to support the present results. A study performed on Atlantic salmon raised from 110 to 340 g at 5°C or 12°C and fed a soybean oil diet reported few effects of temperature on fillet fatty acid composition [3]. In contrast, a significant decrease in EPA and total n-3 LC-PUFA was observed in fillet of Atlantic salmon raised from 160 to 250 g with a vegetable diet and held at 20°C instead of 10°C [4]. Accordingly, a study evaluating the impact of increased temperature on fillet fatty acid composition of rainbow trout raised at 5°C or 19°C reported decreased fillet EPA and DHA contents [5]. Considering SFA and MUFA contents, the absence of pronounced changes in fillet contrasts with the decreased SFA and MUFA contents observed in fillet of juvenile steelhead trout raised with increasing temperatures from 10°C to 18°C and fed a fish oil or linseed oil diet [6]. Conversely, our results on the n-6 PUFA profile of fillet NL and PL are in accordance with this study as unaltered LA and ARA contents in fillet were reported [6].

The adipose tissue is one of the major lipid storage tissues in rainbow trout [7, 8] and may reflect, to some extent, the effects of endogenous lipid bioconversion, which is modulated by external parameters such as water temperature. Since the amounts of n-6 and n-3 LC-PUFA were higher in adipose tissue with the temperature increase, a positive temperature impact on the lipid bioconversion capacity might be drawn as a conclusion. However, the higher feed intake at the increased temperature of 19°C and, therefore, the higher presence of substrates for the bioconversion pathways must be taken in account as an alternative explanation.

It is classically thought that a temperature change primarily affects tissue PL fatty acid composition, whereas a dietary change mainly affects tissue NL, especially in fillet and other lipid storage locations [9-11]. This is at odds with our results in which the fatty acid composition of fillet PL was not affected by temperature. Jobling and Bendiksen [11] also observed only

minor temperature effects on the PL fatty acid composition of Atlantic salmon fillet. These authors concluded that a thermal influence on the PL fatty acid composition may be more pronounced in the mid-to-high end of the fish optimal temperature range than at the lower end [11]. Our data are not consistent with that hypothesis since we did not observe any temperature effect even though the experimental design used 19°C, which is considered as close to the upper limit of the rainbow trout temperature tolerance range [7]. The same authors also pointed out that the thermal influence may be partially obscured by the effects of dietary lipid source replacement, which might have been the case in our study.

Similar conclusions to those reported for whole body may be drawn for the dietary treatment impact on fatty acid composition tissues. Indeed, the fatty acid profile of fillet and adipose tissue reflected that of the diet. This was highlighted by the decrease of 57 % in EPA and 55 % in DHA in the fillet NL of fish fed LO at 15°C. Similar decrease in EPA and DHA contents has been previously observed in the fillet of Atlantic salmon (of 70 % in EPA and 62 % in DHA) [12] and of rainbow trout [13-15] when linseed oil was used as the sole lipid source.

In conclusions, whereas the temperature did not impact the fish tissue fatty acid composition, the dietary lipid source replacement significantly reduced the n-3 LC-PUFA content of both tissues. Research on readily available and economically viable lipid source alternatives to fish oil is thus still required and has likely to be coupled to other feeding strategies in order to maintain the supply of fish rich in health-promoting fatty acids.

### 3.7.4 References


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## 3.8 Description of the whole body fatty acid balance method

The apparent *in vivo* enzymatic activities reported in Table 3.8 were calculated via the implementation of the whole body fatty acid balance method, which has been developed and described in details by Turchini *et al.* [1], with subsequent developments [2, 3]. The computation of the whole body fatty acid balance method involves four steps, which are summarised in the present section in order to help the reader in the comprehension of the Chapters 3 and 4.

Previously to the method implementation, a feeding trial is required to obtain data on initial and final fish body weights and VFI, expressed in g per animal, and on lipid content of fish and diets. Moreover, the quantitative fatty acid composition, expressed in mg per g of lipid, of the diets, initial and final fish is also required. Finally, a digestibility trial is needed to obtain the apparent digestibility coefficient, expressed in %, of each fatty acid for each treatment.

**First step**

The net appearance or disappearance of each fatty acid is determined as the difference between total fatty acid accumulation in fish (= final fatty acid fish content - initial fatty acid fish content) and the fatty acid net intake (= fatty acid intake - fatty acid egestion in faeces), as observed in Figure 3.4. A net fatty acid appearance or disappearance occurs when the difference is positive or negative, respectively. In practice, the fatty acid net intake is obtained by applying the digestibility percentage on the fatty acid ingested content.

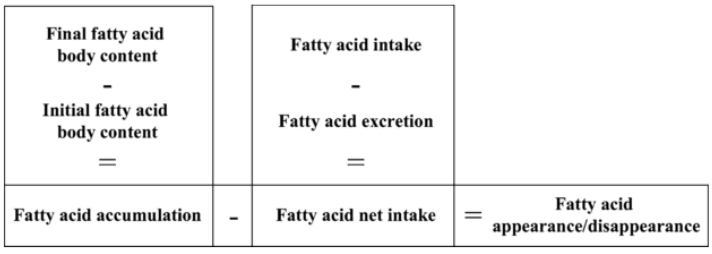


**Figure 3.4.** Schematic representation of the first step calculations of the whole body fatty acid balance method [1].

**Second step**

Using the appearance/disappearance results of each fatty acid, the subsequent step involves a series of backwards calculations along the fatty acid bioconversion pathways of n-3 PUFA, n-6 PUFA, SFA and MUFA. As an example, the calculations within the n-3 PUFA biosynthesis pathway are schematised in Figure 3.5. The results obtained from the first step of the method (fatty acid appeared or disappeared) are represented as δ values (if δ is a negative number, then δ = 0). The φ value (for ALA only) represents the total ALA disappeared, since no appearance is possible in vertebrates. Following a backwards calculation (started from DHA and 22:3n-3), the quantity of each fatty acid converted to longer or more unsaturated homologues is calculated (ε value):

Normal elongation and desaturation pathway (NED)
ε (22:6n-3) = 0
ε (22:5n-3) = δ (22:6n-3)
ε (20:5n-3) = δ (22:5n-3) + ε (22:5n-3)
ε (20:4n-3) = δ (20:5n-3) + ε (20:5n-3)
ε (18:4n-3) = δ (20:4n-3) + ε (20:4n-3)

Elongation towards dead end products (DE)
ε (22:3n-3) = 0
ε (20:3n-3) = δ (22:3n-3)

For ALA:
ε (18:3n-3) = δ (18:4n-3) + ε (18:4n-3) + δ (20:3n-3) + ε (20:3n-3).

**Third step**

For each fatty acid, the quantity β-oxidised, elongated or desaturated can be calculated. The equations are presented with ALA as an example:
ALA elongated to dead end products = δ (20:3n-3) + ε (20:3n-3)
ALA bioconverted through the normal elongation and desaturation pathway = δ (18:4n-3) + ε (18:4n-3)
ALA oxidised = φ 18:3n-3 - ε (18:3n-3).

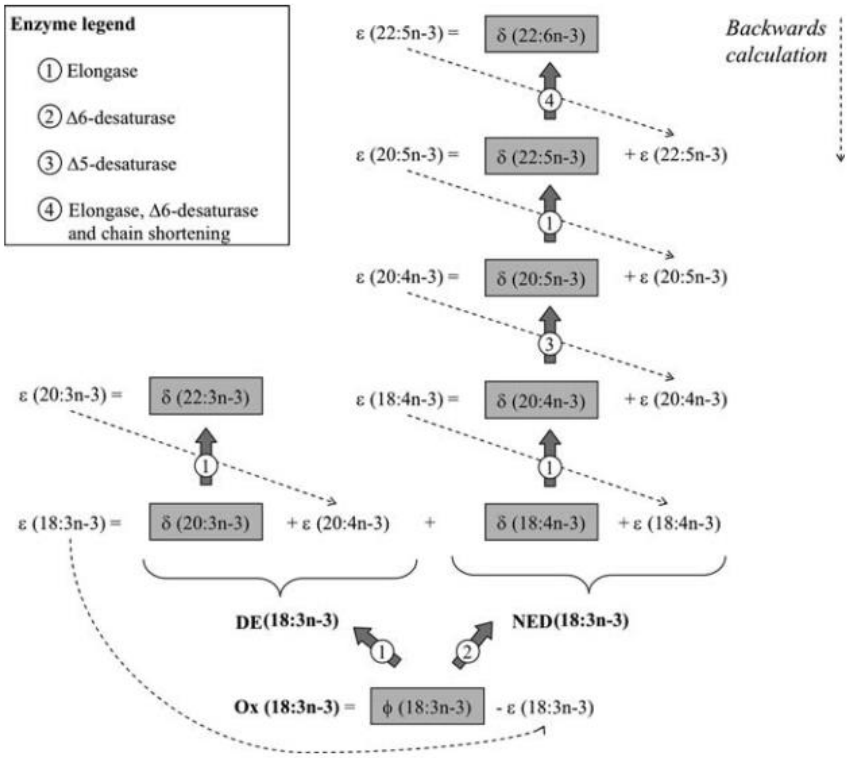


**Figure 3.5.** Schematic representation of the computation involved in the whole-body fatty acid balance method. Shaded figures (δ values) represent the results obtained following the first step of the method, while the φ value (for ALA only) represents the total ALA disappeared (given that no appearance is possible in vertebrates). Via a backwards calculation, starting from the end of the two pathways it is possible to calculate the ε values, which represent the fatty acid converted to longer or more unsaturated homologues. Successively, it is possible to quantify the fate of ALA as (i) chain shortening and oxidation (Ox), (ii) elongation towards a dead end product (DE) or (iii) elongation and desaturation along the normal pathway (NED). Ultimately, it is possible to estimate the elongase, D-5 and D-6 desaturase activities [1].

### Fourth step

The apparent *in vivo* elongation and desaturation enzymatic activities are finally determined and expressed as nmol per g of fish per day. As an example, the apparent *in vivo* enzymatic activities in the n-3 PUFA biosynthesis pathway are obtained through the following equations:

Apparent *in vivo* Δ5 desaturation activity = δ (20:5n-3) + ε (20:5n-3)
Apparent *in vivo* Δ6 desaturation activity = δ (18:4n-3) + ε (18:4 n-3) + δ (22:6 n-3)
Apparent *in vivo* elongation activity = δ (20:3n-3) + δ (20:4n-3) + ε (20:4n-3) + δ (22:5n-3) + ε (22:5n-3) + δ (22:6n-3)

## References

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# Chapter 4

## A n-3 PUFA depletion applied to rainbow trout fry (*Oncorhynchus mykiss*) does not modulate its subsequent lipid bioconversion capacity

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## Foreword

As previously reported in the literature and in Chapter 3, the replacement of fish oil by plant-derived oils induces a decrease of n-3 LC-PUFA in fish tissues. Nutritional strategies are therefore required in order to improve the n-3 LC-PUFA production and retention of fish fed plant-based diets. In the present chapter, the fish fatty acid bioconversion capacity has been evaluated with rainbow trout fry previously depleted in n-3 PUFA. We hypothesised that a high n-3 PUFA depletion of fish could improve their bioconversion capacity when feeding a ALA-rich diet. For that purpose, fish fed a OLA-rich sunflower oil diet (SO) during a 60-day pre-experimental period and subsequently fed either a ALA-rich linseed oil diet (LO) (this treatment being called SO/LO) or a EPA and DHA-rich fish oil diet (FO) (this treatment being called SO/FO) during a 36-day experimental period were compared to fish continuously fed SO, LO or FO throughout the feeding trial (96 days). The experimental design is schematised in Figure 4.1. The present results have been submitted to the British Journal of Nutrition and are currently under review. The digestibility results, which are not included in the article, are presented as additional data.

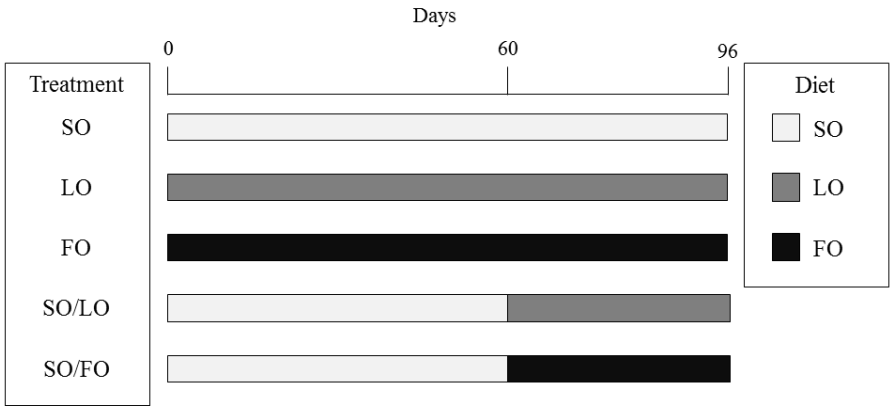


**Figure 4.1.** Experimental design used in Chapter 4 to evaluate the impact of a n-3 PUFA depletion of fish on its subsequent fatty acid bioconversion capacity. Rainbow trout fry were depleted in n-3 PUFA through a pre-experimental feeding period (from day 0 to day 60) with a sunflower oil-based diet (SO). This was followed by an experimental period (from day 61 to day 96) during which fish were fed either a linseed oil-based diet (LO) (this treatment being called SO/LO) or a fish oil-based diet (FO) (this treatment being called SO/FO). These treatments were compared to fish continuously fed on SO, LO or FO throughout the feeding trial (from day 0 to day 96).

## Abstract

Nutritional strategies are currently developed to produce farmed fish rich in *n*-3 long-chain PUFA (LC-PUFA) whilst replacing fish oil by plant-derived oils in aquafeeds. The optimisation of such strategies requires a thorough understanding of fish lipid metabolism and its nutritional modulation. The present study evaluated the fatty acid bioconversion capacity of rainbow trout (*Oncorhynchus mykiss*) fry previously depleted in *n*-3 PUFA through a 60-day pre-experimental feeding period with a sunflower oil-based diet (SO) followed by a 36-day experimental period during which fish were fed either a linseed oil-based diet (LO) (this treatment being called SO/LO) or a fish oil-based diet (FO) (this treatment being called SO/FO). These treatments were compared to fish continuously fed on SO, LO or FO for 96 d. At the end of the 36-day experimental period, SO/LO and SO/FO fish recovered more than 80 % of the *n*-3 LC-PUFA reported for LO and FO fish, respectively. Fish fed LO showed high apparent *in vivo* elongation and desaturation activities along the *n*-3 biosynthesis pathway. However, at the end of the experimental period, no impact of the fish *n*-3 PUFA depletion was observed on apparent *in vivo* elongation and desaturation activities of SO/LO fish as compared to LO fish. In contrast, the fish *n*-3 PUFA depletion negatively modulated the *n*-6 PUFA bioconversion capacity of fish in terms of reduced apparent *in vivo* elongation and desaturation activities. The effects were similar after 10 or 36 d of the experimental period, indicating the absence of short-term effects.



## Keywords

Rainbow trout - Fatty acid metabolism - Lipid bioconversion capacity - Plant-derived oils - Whole-body fatty-acid-balance method

## 4.1 Introduction

There is an expectation on aquaculture to supply fish rich in health promoting *n*-3 long-chain PUFA (*n*-3 LC-PUFA), principally EPA (20:5*n*-3) and DHA (22:6*n*-3). It is well established that *n*-3 LC-PUFA impart a host of positive effects on human health [1; 2; 3]. Moreover, *n*-3 LC-PUFA are essential fatty acids for the optimal growth and health of fish[4; 5]. Typically, the high *n*-3 LC-PUFA content in farmed fish is derived from the inclusion of marine-derived fish oil as one of the dietary lipid sources within aquafeeds[6; 7]. However, fish oil has become expensive and difficult to source, and given its status as a finite marine resource, its utilisation is widely criticised from a sustainability perspective[6; 7]. One of the key sustainable alternatives to fish oil are plant-derived oils[8]. In contrast to fish oil, plant-derived oils lack LC-PUFA[8] but are particularly rich in MUFA and $C_{18}$ PUFA, especially 18:1*n*-9 and linoleic acid (LA, 18:2*n*-6), and in certain sources, such as linseed, camelina or perilla oils, rich in alpha-linolenic acid (ALA, 18:3*n*-3)[5; 8].

Among fish species, rainbow trout (*Oncorhynchus mykiss*) and other salmonids possess a relatively high capacity to endogenously convert the dietary essential fatty acids LA and ALA into *n*-6 and *n*-3 LC-PUFA through a combination of desaturation steps requiring Δ-6 and Δ-5 desaturases, elongation steps requiring elongases 2 and 5, and partial β-oxidation[4; 9; 10; 11; 12; 13]. Previous studies reported increased desaturation and elongation activities without significant detrimental effects to growth and health in salmonids fed plant-derived oil diets (*i.e.* sunflower oil[14; 15; 16], olive oil[14], palm oil[16], rapeseed oil[16; 17] or linseed oil[14; 15; 17; 18]), as a blend or sole source. In rainbow trout, the complete dietary replacement of fish oil by linseed oil stimulated fatty acid metabolism along the bioconversion pathway[18; 19; 20; 21]. However, while providing a highly suitable source of energy for fish growth and maintenance, it is well documented that the fatty acid composition of the dietary lipid source is reflected in fish tissues. Therefore, despite an increase in the bioconversion capacity, fish fed plant-based diets invariably contained lower EPA and DHA concentrations as compared to those fed fish oil diets[15; 16; 18; 19; 20; 21], resulting in major drawbacks from a fish consumption perspective.

There is currently a need to optimise feeding strategies to facilitate the production of farmed fish rich in *n*-3 LC-PUFA whilst minimising fish oil inclusion in aquafeeds. Finishing diets, given prior to harvest and formulated

with fish oil, have been investigated to restore the *n*-3 LC-PUFA content in fish previously fed plant-based diets throughout the grow-out period. Previous studies have demonstrated positive results in many fish species, including Atlantic salmon (*Salmo salar*)[22; 23], common carp (*Cyprinus carpio*)[24], red hybrid tilapia (*Oreochromis sp.*)[25], European sea bass (*Dicentrarchus labrax*)[26], red seabream (*Pagrus auratus*)[27] and Murray cod (*Maccullochella peelii peelii*)[28]. An EPA and DHA recovery rate of approximately 80 % was reported at the end of the finishing period in Atlantic salmon previously fed plant-based diets[22; 23]. In rainbow trout, finishing diets induced a shift in fish fatty acid profiles to a more fish oil-like composition, but were unable to achieve similar *n*-3 LC-PUFA concentrations as compared to fish fed fish oil throughout their growth[19; 29]. The efficiency of a finishing period is determined by a combination of factors including the fish species, the finishing period duration, the fatty acid profile of the alternative oil used[19; 25; 28] (i.e. the dietary $C_{18}$ PUFA level[30]), or the application of a short term feed deprivation period before the commencement of the finishing period[29]. While the various feeding strategies that incorporate a finishing strategy demonstrate promising results with undoubtable positive environmental and economic effects, they still rely upon the inclusion of unsustainable dietary fish oil. An alternative strategy involves stimulating fish fatty acid metabolism through nutritional programming during early larval stages as a means of improving the acceptance and conversion of dietary ALA from plant-based diets at juvenile stages[31; 32; 33]. Vagner *et al.*[31] observed increased Δ-6 desaturase gene expression in European sea bass juveniles fed an *n*-3 LC-PUFA deficient diet from day 83 post-hatch to day 118, when larvae had been previously fed a low *n*-3 LC-PUFA diet (0.5 % EPA + DHA), as compared to a high *n*-3 LC-PUFA diet (3.7 %). Moreover, in a study where rainbow trout were fed a plant-based diet containing deuterated ALA, a higher conversion of dietary deuterated ALA to DHA was observed in smaller fish (0.5 to 1.5 g) in comparison to larger fish (6 to 8 g), highlighting the rapid change of bioconversion capacity with fish size[34]. Collectively, the results of these studies provide promising insight into the implementation of feeding strategies for the optimisation of EPA and DHA production and retention in fish tissues. However, for the most part these strategies have not been tested in unison, yielding positive yet incremental benefits. To date, the impacts of combined strategies for increasing *n-3* LC-PUFA deposition currently remain unknown, ultimately requiring dedicated assessment to determine the extent to which utilisation measures can be optimised.

The aim of the present study was to evaluate the fatty acid bioconversion capacity of rainbow trout fry previously depleted in *n*-3 PUFA through feeding on a sunflower oil-based diet (SO) during a 60-day pre-experimental period and subsequently fed either a ALA-rich linseed oil diet (LO) or a EPA and DHA-rich fish oil diet (FO) in a 36-day experimental period. Fish growth and bioconversion capacity were evaluated at the end of both periods and on the 10$^{th}$ day of the experimental period, in order to determine the potential impact of a *n*-3 PUFA fish depletion on the apparent *in vivo* elongation and desaturation activities in fish fed ALA. Three additional control groups included fish fed on SO (*n*-3 PUFA deficient diet), LO (ALA-rich diet) or FO (EPA and DHA-rich diet) throughout the feeding trial.

## 4.2 Experimental procedures

### 4.2.1 Ethics statement

The experimental design of the feeding and digestibility trials were approved by the Animal Care and Use Committee of the Université catholique de Louvain (Permit number 103203) as per the EU legal frameworks relating to the protection of animals used for scientific purposes (Directive 86/609/CEE) and guidelines of Belgian legislation governing the ethical treatment of animals (Decree M.B. 05.01.1994, November 14th, 1993). Both *in vivo* experiments were conducted at the "Plateforme technologique et didactique en biologie aquicole Marcel Huet" (Université catholique de Louvain, Louvain-la-Neuve, Belgium), which is certified for animal services under the permit number LA 1220034. All manipulations were performed under anaesthesia and, if necessary, fish were euthanised using 2-phenoxyethanol at the required concentrations. All efforts were made to minimise fish numbers and suffering. No clinical symptoms were observed within or outside the experimental periods.

### 4.2.2 Experimental diets

Experimental diets were formulated to differ in their fatty acid composition and contained either a high amount of 18:1*n*-9 for SO (blend of sunflower oils rich and low in 18:1*n*-9, 87:13, *v:v*), ALA for LO or *n*-3 LC-PUFA for FO. All diets were formulated to cover the fish requirement in LA, while avoiding any excess in that fatty acid, which might compete with ALA regarding desaturations and elongations. In practice, 18:1*n*-9-poor sunflower oil was included to all experimental diets (5 g/kg of dry matter (DM)). A

higher inclusion of 18:1*n*-9-poor sunflower oil was used for the SO diet (15 g/kg DM as compared to 5 g/kg DM) in order to obtain a similar LA content in the SO and LO diets. In addition, a sunflower oil rich in 18:1*n*-9 and poor in LA was added to the SO diet at a 65 g/kg DM concentration to obtain a similar oil inclusion between all experimental diets. The experimental diets were formulated to meet the protein, vitamin and mineral requirements of rainbow trout[5; 35] (Table 4.1). The SO, LO and FO diets had a crude fat content of 94.1, 90.4, and 94.9 mg/g of dry matter (DM), respectively. This lipid content level was chosen in order to obtain a quick and efficient depletion in *n*-3 PUFA in the fish submitted to the SO treatment. Moreover, diets were formulated to obtain a targeted crude protein content of 520 mg/g DM and a targeted energy content of 20 MJ/kg DM. SO was deficient in *n*-3 PUFA (1.2 % of identified fatty acids), whereas LO was particularly rich in ALA (39.3 % of identified fatty acids, 99.8 % of *n*-3 PUFA), and FO rich in EPA and DHA (7.6 and 9 % of identified fatty acids, 33.7 % and 40 % of *n*-3 PUFA, respectively) (Table 4.2). Chromic oxide was added at 10 g/kg DM to each experimental diets intended for the digestibility trial in order to serve as indigestible marker. The dry dietary components were mixed, homogenised using an electronic mixer (Kenwood Ltd., Havant, Hants, UK), extruded (HI 2251, Simplex, Paris, France). The diets were subsequently freeze-dried, manually crushed and then sieved to obtain pellets from 0.8 to 1.6 mm. The dry pellets were finally coated with oils and the diets were shaken several times for 48 h at 4°C prior to storage at -20°C until feeding or analysis.

**Table 4.1.** Components (g/kg DM) of the experimental diets formulated with sunflower oil (SO), linseed oil (LO), or cod liver oil (FO).

| | SO | LO | FO |
|---|---|---|---|
| Casein [*] | 288.3 | 288.3 | 288.3 |
| Gelatin [*] | 50 | 50 | 50 |
| Wheat gluten meal [*] | 250 | 250 | 250 |
| Modified starch [*] | 161.7 | 161.7 | 161.7 |
| Glucose [*] | 25 | 25 | 25 |
| Agar [*] | 10 | 10 | 10 |
| Carboxymethylcellulose [*] | 40 | 40 | 40 |
| Cellulose [*] | 20 | 20 | 20 |
| Vitamin premix [*, †] | 10 | 10 | 10 |
| Mineral premix [*, ‡] | 65 | 65 | 65 |
| 18:1n-9 rich sunflower oil [§] | 65 | 0 | 0 |
| 18:1n-9 poor sunflower oil [§] | 15 | 5 | 5 |
| Linseed oil [‖] | 0 | 75 | 0 |
| Cod liver oil [¶] | 0 | 0 | 75 |

[*] Sigma-Aldrich, St-Louis, MO, USA.

[†] Vitamin complex (g/kg premix) according to Rollin *et al.* [(32)]: retinol acetate 0.67, ascorbic acid 120, cholecalciferol 0.1, α-tocopherol acetate 34.2, menadione 2.2, thiamin 5.6, riboflavin 12, pyridoxine 4.5, calcium pantothenate 14.1, p-aminobenzoic acid 40, cyanocobalamin 0.03, niacin 30, biotin 0.1, choline chloride 350, folic acid 1.5, inositol 50, canthaxanthin 10, butylated hydroxytoluene 1.5, butylated hydroxyanisole 1.5, α-cellulose 322.1.

[‡] Mineral complex (g/kg premix) according to Rollin *et al.* [(32)]: $CaHPO_4.2H_2O$ 295.5, $Ca(H_2PO_4)_2.H_2O$ 217, $NaHCO_3$ 94.5, $Na_2SeO_3 \cdot 5H_2O$ 0.011, KCl 100, NaCl 172.4, KI 0.2, $MgCl_2$ 63.7, $MgSO_4.7H_2O$ 70.32, $MnSO_4.H_2O$ 1.52, $FeSO_4.7H_2O$ 12.41, $CuSO_4.5H_2O$ 0.4, $ZnSO_4.7H_2O$ 10.

[§] Vandemoortele, Izegem, Belgium.

[‖] Lambert chemicals, Herstal, Belgium.

[¶] Certa, Waregem, Belgium.

**Table 4.2.** Fatty acid composition (mg/g DM) of the experimental diets.

| Fatty acids | SO | LO | FO |
|---|---|---|---|
| 14:0 | 0.1 | 0.1 | 2.7 |
| 16:0 | 4.9 | 5.7 | 9.0 |
| 18:0 | 2.0 | 2.4 | 1.4 |
| 16:1*n*-7 | 0.1 | 0.1 | 3.4 |
| 18:1*n*-7 | 1.0 | 0.7 | 1.9 |
| 18:1*n*-9 | 48.4 | 13.2 | 11.6 |
| 20:1*n*-9 | 0.2 | 0.1 | 4.4 |
| 18:2*n*-6 | 15.9 | 17.6 | 9.7 |
| 18:3*n*-6 | / | 0.1 | 0.04 |
| 20:2*n*-6 | / | 0.02 | 0.2 |
| 20:3*n*-6 | / | 0.02 | 0.05 |
| 20:4*n*-6 | / | / | 0.2 |
| 22:4*n*-6 | / | / | / |
| 22:5*n*-6 | / | / | 0.1 |
| 18:3*n*-3 | 0.9 | 26.3 | 1.2 |
| 18:4*n*-3 | / | / | 0.9 |
| 20:3*n*-3 | / | 0.1 | 0.1 |
| 20:4*n*-3 | / | / | 0.5 |
| 20:5*n*-3 | / | / | 4.5 |
| 22:5*n*-3 | / | / | 0.8 |
| 22:6*n*-3 | / | / | 5.3 |
| Total | 74.4 | 66.9 | 59.1 |
| Σ SFA [*] | 7.7 | 8.5 | 13.5 |
| Σ MUFA [†] | 49.9 | 14.3 | 21.9 |
| Σ $C_{18}$ *n*-6 PUFA [‡] | 15.9 | 17.8 | 9.7 |
| Σ *n*-6 LC-PUFA [§] | 0 | 0.04 | 0.6 |
| Σ $C_{18}$ *n*-3 PUFA [‖] | 0.9 | 26.3 | 2.1 |
| Σ *n*-3 LC-PUFA [¶] | 0 | 0.1 | 11.2 |
| *n*-3/*n*-6 [**] | 0.1 | 1.5 | 1.3 |
| *n*-3/*n*-6 LC-PUFA [††] | 0 | 1.2 | 18.9 |

SO, sunflower oil-based diet; LO, linseed oil-based diet; FO, fish oil-based diet.

[*] Sum of SFA, includes 20:0, 22:0 and 24:0.

[†] Sum of MUFA, includes 14:1n-5, 22:1n-9 and 24:1n-9.

[‡] Sum of *n*-6 PUFA with 18 C.

[§] Sum of *n*-6 long-chain PUFA with 20 C and 22 C.

[‖] Sum of *n*-3 PUFA with 18 C.

[¶] Sum of *n*-3 long-chain PUFA with 20 C and 22 C.

[**] Ratio of total *n*-3 PUFA to total *n*-6 PUFA.

[††] Ratio of *n*-3 long-chain PUFA to *n*-6 long-chain PUFA.

### 4.2.3 Fish husbandry

Fertilised eggs from domesticated rainbow trout breeders were supplied by a commercial fish farm (La Fontaine aux Truites, Gérouville, Belgium). After hatching, rainbow trout fry were fed a commercial diet for 2 months prior to the feeding trial. After 48 h of feed deprivation, rainbow trout fry (mean initial body weight 0.70 (SEM 0.01) g/fish) were randomly distributed among 17 tanks (11 litres capacity) to obtain 225 fish per tank. Fish of two tanks were sampled as an initial sample, weighed, and stored at -20°C for subsequent analyses. Throughout the feeding trial, feeding was carried out by hand twice daily (08.30 and 16.00) to apparent satiation (pellets from 0.8 to 1.6 mm, depending on the fish size). Fish were subjected to a 12:12 h light:dark cycle photoperiod at a mean water temperature of 14°C with a 1 l/min flow. From the first to the 60$^{th}$ feeding day, fish of nine tanks were fed on SO ($n$ = 9), three tanks were fed on LO ($n$ = 3) and three tanks were fed on FO ($n$ = 3). On the 20$^{th}$ day, fish were transferred to larger tanks (50 litre capacity) supplied by water at 11.5 ± 0.5°C on a 5 l/min flow basis. At the end of the 60-day pre-experimental period, considered to be long enough to highly reduce the *n*-3 PUFA content of fish fed on SO, six tanks previously held on SO were switched, either to LO (three tanks), or FO (three tanks). The second feeding period lasted 36 d. The experimental conditions were therefore named as SO, LO and FO for fish fed on SO, LO and FO ($n$ = 3), respectively, during 96 d, and as SO/LO and SO/FO ($n$ = 3) for fish fed on SO during the first 60-day pre-experimental period and then on LO or FO, respectively, during the second 36-day experimental period. Throughout the feeding trial, the biomass was determined every 10 feeding d after 48 h of feed deprivation. On days 60, 70 and 96, fish were weighed after 48 h of feed deprivation and 15 fish of each tank were then euthanised with 2-phenoxyethanol, freeze-dried, homogenised and kept frozen (-20°C) until chemical analysis. At the end of the experimental period, the remaining fish from each tank fed their specific diet until the digestibility trial. The digestibility trial was performed with 5 ± 0.05 kg of fish in circular tanks (130 litre capacity). Fish remained under experiment until accumulating sufficient faeces. The water was supplied at a 4 l/min flow, the temperature was maintained at 11 ± 1°C throughout the trial and fish were subjected to a 12:12 h light:dark cycle photoperiod. Fish were fed manually twice daily (09.00 and 17.00) to apparent satiation whilst avoiding any undesirable mixing of feed and faeces. Faeces were collected continuously through a rotating automatic faeces collector system[36]. Faeces collected per tank were

weighed, freeze-dried, homogenised and stored at -20°C until further analyses.

### 4.2.4 Chemical analysis

The DM and crude fat contents were analysed following analytical methods from the Association of Official Analytical Chemists[37]. Briefly, DM was measured by drying samples at 105°C for 16 h and the crude fat content was evaluated using diethyl ether extraction according to Soxhlet method. The chromic oxide concentration in diets and faeces was determined following a protocol involving acid digestion followed by oxidation prior to photometric measurement (Cecil Instruments, Cambridge, UK) at 350 nm[38]. The fatty acid composition of diets, fish and faeces was evaluated after lipid extraction of samples following the Folch method[39] with subsequent modifications[40]. Briefly, lipids from 1 g of dried sample were extracted by 60 ml of chloroform/methanol (2:1, *v:v*) (VWR chemicals, Radnor, PA, USA). Tridecanoic acid (Sigma-Aldrich) was used as internal standard for fatty acid quantification. The extracted fatty acids were converted into fatty acid methyl esters via methylation under alkaline conditions (KOH in methanol, 0.1 M, at 70°C for 60 min) and then under acidic conditions (HCl in methanol, 1.2 M, at 70°C for 20 min). The resultant fatty acid methyl esters were subsequently separated by gas chromatography. The GC Trace (Thermo Scientific, Milan, Italy) was equipped with an RT2560 capillary column (100 m × 0.25 mm internal diameter, 0.2 µm film thickness) (Restek, Bellefonte, PA, USA), an "on column" automatic injector and a flame ionisation detector kept at a constant temperature of 255°C. The system used hydrogen as the carrier gas at an operating pressure of 200 kPa. The oven temperature program was as follows: an initial temperature of 80°C, which progressively increased at 25°C/min up to 175°C, a holding temperature of 175°C during 25 min followed by an increase at 10°C/min up to 205°C, a holding temperature of 205°C during 4 min followed by an increase at 10 C/min up to 225°C and a holding temperature of 225°C during 20 min. Each peak was identified by comparison of retention times with those for pure methyl ester standards (Larodan (Solna, Sweden) and Nu-Check Prep (Elysian, USA)). Data processing was operated via ChromQuest software 3.0 (Thermo Finnigan, Milan, Italy). The final results are expressed in mg/g of DM.

### 4.2.5 Performance parameters and fatty acid metabolism computation

Daily growth coefficient (DGC) was calculated as follows: DGC (($g^{1/3}$/d) ×1000) = 1000 ×[(final fish weight (g))$^{1/3}$ – (initial fish weight (g))$^{1/3}$] / feeding d. Daily feed intake was calculated as the percentage of biomass. Feed efficiency (FE, g/g DM) was calculated as the ratio between fish weight gain (g) and dry feed intake (g DM). The apparent fatty acid digestibility was assessed using the standard formula: 100 – [100 × ($Cr_2O_3$ in diet (mg/g DM)) / ($Cr_2O_3$ in faeces (mg/g DM)) × (fatty acid in faeces (mg/g DM)) / (fatty acid in diet (mg/g DM))]. The coefficient of distance (CD) was implemented to compare fatty acid concentrations between two treatments and was calculated as previously described[41]. The estimation of the apparent *in vivo* fatty acid metabolism was calculated via the implementation of the whole body fatty acid balance method, as initially proposed and described by Turchini *et al*.[42] and later modified[20; 43].

### 4.2.6 Statistical analysis

All data are presented as means with their standard errors ($n$ = 2, 3 or 9, as stated). Prior to statistical analysis, data were subjected to log or square root transformation if identified as non-homogenous (Levene's test) to meet the assumptions for statistical methods. The significance of difference between dietary treatments was determined using one-way ANOVA at a significance level of $\alpha$ 5 %, followed by Tukey's (parametric with $\alpha$ 5 %) or Wilcoxon's (nonparametric with $\alpha$ 1.69 %) *post hoc* tests. Statistical analysis was carried out using JMP® Pro 12 (SAS, Cary, NC, USA).

## 4.3 Results

### 4.3.1 Fish growth performance

The experimental diets were readily accepted by fish and mortality throughout the feeding trial was low and unrelated to the dietary treatment (mean mortality rate lower than 0.01% per d). In contrast, fish weight and growth performance were highly impacted by the dietary lipid source. Fish fed on SO throughout the feeding trial recorded the lowest final weight (22.9 (SEM 0.9) g/fish) whereas fish fed on LO and FO recorded the highest final weights (48.4 (SEM 1.2) and 51.5 (SEM 0.9) g/fish, respectively) (Figure 4.2). This trend manifested further in decreased DGC and FE in fish

subjected to the SO treatment over the course of the feeding trial (Table 4.3). In LO fish, a reduced DGC was noticed in comparison to fish fed on FO at the end of the 60-day pre-experimental period, but not at the end of the feeding trial. The replacement of SO by LO or FO for 36 d also induced significant differences. The SO/LO and SO/FO final fish weights were higher than those of fish fed on SO for 96 d, but did not reach those of fish constantly fed on LO and FO for 96 d (Figure 4.2). DGC values were also higher for the SO/LO and SO/FO treatments as compared to the SO treatment, and similar to those observed for the LO and FO fish groups (Table 4.3). Moreover, an increased FE was recorded for SO/LO and SO/FO fish as compared to SO fish. These increased FE were similar to those of fish fed on LO and FO for 96 d. The SO/LO fish had a significantly reduced DGC as compared to the SO/FO fish group but similar final fish weights, feed intake and FE.

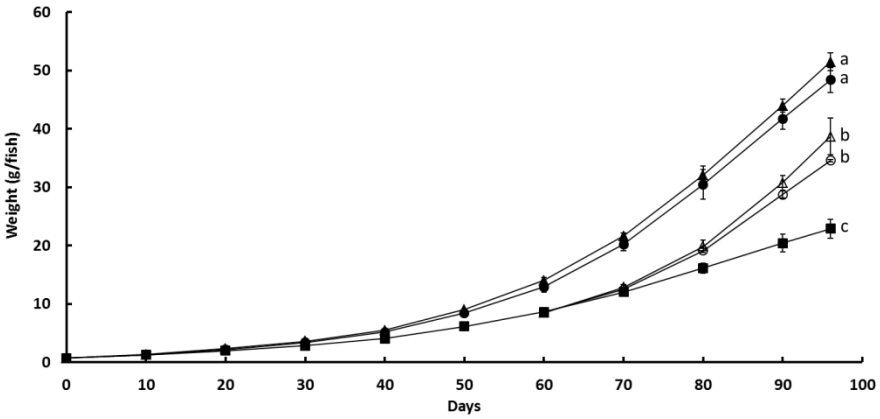


**Figure 4.2.** Mean weight (g/fish) of rainbow trout at each sampling time point over the 60-day pre-experimental period and the 36-day experimental period. Values are means ($n$ = 3 except SO treatment from starting day until day 60 for which $n$ = 9), with their standard errors represented by vertical bars. The fish weight was impacted by the dietary treatment regarding the lowest weight of fish fed sunflower oil (SO, ■) and the highest weights of fish fed linseed oil (LO, ●) or fish oil (FO, ▲) during 96 d. Intermediate fish weights were reported when feeding fish on SO for 60 d and then on LO (SO/LO, ○) or FO (SO/FO, Δ) for 36 d. $^{a,b,c}$ Mean values with no common superscript letter were significantly different (Tukey's *post hoc* test, $\alpha$ 5 %).

**Table 4.3.** Growth performance of rainbow trout fed diets differing in fatty acid composition during a 60-day pre-experimental feeding period (day 1 to 60) followed by a 36-day experimental period (days 61 to 96)
(Mean values with their standard error of the mean; $n = 3$ except for SO for day 1 to 60 period ($n = 9$))

| | SO | | LO | | FO | | SO/LO | | SO/FO | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| **Days 1 to 60** | | | | | | | | | | |
| DGC (($g^{1/3}$/d) × 1000) | 19.3$^{c}$ | 0.1 | 24.2$^{b}$ | 0.5 | 25.4$^{a}$ | 0.3 | / | / | / | / |
| Feed intake (%/d) | 2.9$^{b}$ | 0.0 | 3.2$^{a}$ | 0.1 | 3.1$^{a}$ | 0.0 | / | / | / | / |
| FE | 1.4$^{b}$ | 0.0 | 1.5$^{a,b}$ | 0.1 | 1.6$^{a}$ | 0.0 | / | / | / | / |
| **Days 1 to 96** | | | | | | | | | | |
| DGC (($g^{1/3}$/d) × 1000) | 20.3$^{b}$ | 0.4 | 28.7$^{a}$ | 0.3 | 29.5$^{a}$ | 0.2 | / | / | / | / |
| Feed intake (%/d) | 2.8$^{b}$ | 0.0 | 3.0$^{a}$ | 0.1 | 2.9$^{a,b}$ | 0.0 | / | / | / | / |
| FE | 1.1$^{b}$ | 0.0 | 1.3$^{a}$ | 0.0 | 1.4$^{a}$ | 0.0 | / | / | / | / |
| **Days 61 to 96** | | | | | | | | | | |
| DGC (($g^{1/3}$/d) × 1000) | 21.9$^{c}$ | 0.7 | 36.1$^{a,b}$ | 0.0 | 36.4$^{a,b}$ | 0.9 | 33.7$^{b}$ | 0.1 | 37.3$^{a}$ | 1.2 |
| Feed intake (%/d) | 2.5$^{c}$ | 0.0 | 2.7$^{b,c}$ | 0.0 | 2.5$^{c}$ | 0.1 | 2.8$^{a,b}$ | 0.0 | 2.9$^{a}$ | 0.0 |
| FE | 1.0$^{b}$ | 0.0 | 1.3$^{a}$ | 0.0 | 1.4$^{a}$ | 0.0 | 1.3$^{a}$ | 0.0 | 1.4$^{a}$ | 0.0 |

SO, sunflower oil-based diet; LO, linseed oil-based diet; FO, fish oil-based diet; SO/LO, SO until day 60 and then LO from days 61 to 96; SO/FO, SO until day 60 and then FO from days 61 to 96; DGC, daily growth coefficient; FE, feed efficiency. $^{a,b,c}$ Mean values within a row with no common superscript letter were significantly different (Tukey's (parametric, $\alpha$ 5 %) or Wilcoxon's (nonparametric, $\alpha$ 1.69 %) *post hoc* tests).

### 4.3.2 Fish fatty acid composition

Throughout the feeding trial, fish fed on LO recorded the highest $C_{18}$ *n*-6 PUFA concentration while fish fed on SO recorded the highest *n*-6 LC-PUFA concentration (Tables 4.4 & 4.5), despite the absence of *n*-6 LC-PUFA in SO (Table 4.2). The pre-experimental period induced a high *n*-3 PUFA depletion for fish fed on SO since these recorded the lowest concentrations of $C_{18}$ *n*-3 PUFA and *n*-3 LC-PUFA (0.79 (SEM 0.10) and 2.75 (SEM 0.47) mg/g DM throughout the feeding trial, respectively). In contrast, the highest $C_{18}$ *n*-3 PUFA and *n*-3 LC-PUFA concentrations were, respectively, reported in fish fed on LO (42.68 (SEM 0.74) mg/g DM) and in fish fed on FO (25.82 (SEM 0.94) mg/g DM) (Tables 4.4 & 4.5). Concentrations of 18:4*n*-3, 20:3*n*-3 and 20:4*n*-3 were significantly higher in fish fed on LO, while fish fed on FO recorded the highest EPA and DHA concentrations. On the $10^{th}$ day of the experimental period (day 70), the SO/LO fish recovered 57 % (CD 3.9) of the *n*-3 LC-PUFA found in fish fed on LO for 70 d. Similarly, the SO/FO fish recovered 51 % (CD 9) of the *n*-3 LC-PUFA found in fish fed on FO for 70 d (Table 4.5). At the end of the experimental period, the *n*-3 LC-PUFA of SO/LO and SO/FO fish almost reached those of fish fed on LO and FO with a recovery rate of 82 % (CD 1.8) and 84 % (CD 2.9), respectively. In terms of DHA, the SO/LO fish recovered 62 % (CD 3.4) and 84 % (CD 1.6) of the DHA found in fish fed on LO on the $10^{th}$ day and at the end of the 36-day experimental period, respectively. The SO/FO fish recovered 49 % (CD 8.6) and 85 % (CD 2.8) of the DHA found in fish fed on FO, on the $10^{th}$ day and at the end of the experimental period, respectively.

**Table 4.4.** Fatty acid composition (mg/g DM) of fish held on dietary treatments differing in the dietary lipid source on the starting and at the end of the 60-day pre-experimental feeding period

(Mean values with standard error of the mean; $n = 3$ except initial treatment ($n = 2$))

| | | | Day 60 | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Initial | | SO | | LO | | FO | |
| Fatty acids | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 14:0 | 7.65 | 0.18 | 2.83 [b] | 0.01 | 2.30 [c] | 0.09 | 7.08 [a] | 0.16 |
| 16:0 | 26.75 | 0.67 | 30.79 [c] | 0.08 | 33.12 [b] | 0.36 | 39.01 [a] | 1.21 |
| 18:0 | 4.74 | 0.39 | 8.31 [b] | 0.07 | 10.26 [a] | 0.26 | 8.05 [b] | 0.36 |
| 16:1*n*-7 | 8.09 | 0.17 | 10.00 [b] | 0.09 | 9.72 [b] | 0.27 | 15.90 [a] | 0.56 |
| 18:1*n*-7 | 4.08 | 0.04 | 4.28 [b] | 0.10 | 3.79 [b] | 0.10 | 6.82 [a] | 0.22 |
| 18:1*n*-9 | 17.51 | 0.53 | 124.74 [a] | 0.89 | 59.00 [b] | 0.92 | 49.44 [c] | 1.20 |
| 20:1*n*-9 | 8.70 | 0.18 | 4.36 [b] | 0.07 | 2.00 [c] | 0.02 | 9.78 [a] | 0.37 |
| 18:2*n*-6 | 5.08 | 0.26 | 25.37 [b] | 0.33 | 36.33 [a] | 0.81 | 22.91 [b] | 0.74 |
| 18:3*n*-6 | 0.13 | 0.00 | 2.77 [a] | 0.05 | 1.23 [b] | 0.09 | 0.43 [c] | 0.01 |
| 20:2*n*-6 | 0.48 | 0.00 | 1.25 [b] | 0.03 | 1.58 [a] | 0.05 | 1.54 [a] | 0.07 |
| 20:3*n*-6 | 0.25 | 0.01 | 2.32 [a] | 0.02 | 1.69 [b] | 0.07 | 1.04 [c] | 0.01 |
| 20:4*n*-6 | 1.30 | 0.02 | 3.38 [a] | 0.02 | 0.97 [b] | 0.03 | 1.03 [b] | 0.01 |
| 22:4*n*-6 | 0.05 | 0.01 | 0.41 [a] | 0.02 | 0.06 [b] | 0.00 | 0.08 [b] | 0.01 |
| 22:5*n*-6 | 0.29 | 0.05 | 3.24 [a] | 0.07 | 0.20 [c] | 0.01 | 0.32 [b] | 0.01 |
| 18:3*n*-3 | 2.47 | 0.20 | 0.64 [c] | 0.05 | 36.27 [a] | 0.74 | 2.15 [b] | 0.06 |
| 18:4*n*-3 | 2.72 | 0.13 | 0.35 [c] | 0.01 | 4.94 [a] | 0.33 | 1.23 [b] | 0.02 |
| 20:3*n*-3 | 0.62 | 0.04 | 0.00 [c] | 0.00 | 1.66 [a] | 0.10 | 0.40 [b] | 0.03 |
| 20:4*n*-3 | 1.53 | 0.07 | 0.06 [c] | 0.00 | 2.11 [a] | 0.09 | 1.00 [b] | 0.06 |
| 20:5*n*-3 | 10.62 | 0.25 | 0.58 [c] | 0.02 | 2.31 [b] | 0.09 | 4.61 [a] | 0.11 |
| 22:5*n*-3 | 2.55 | 0.05 | 0.15 [c] | 0.01 | 0.79 [b] | 0.02 | 1.49 [a] | 0.05 |

**Table 4.4** - Continued

| | Initial | | Day 60 | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | SO | | LO | | FO | |
| Fatty acids | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 22:6*n*-3 | 30.57 | 0.76 | 2.67 [c] | 0.00 | 10.33 [b] | 0.23 | 17.77 [a] | 0.62 |
| Total | 139.94 | 4.05 | 230.97 [a] | 1.44 | 222.07 [a] | 3.55 | 195.42 [b] | 5.82 |
| Σ SFA [*] | 39.54 | 1.24 | 43.37 | 0.10 | 46.32 | 0.21 | 54.67 | 1.75 |
| Σ MUFA [†] | 41.74 | 0.96 | 144.40 [a] | 1.05 | 75.28 [c] | 1.22 | 84.76 [b] | 2.44 |
| Σ $C_{18}$ *n*-6 PUFA [‡] | 5.21 | 0.26 | 28.14 [b] | 0.29 | 37.56 [a] | 0.88 | 23.33 [c] | 0.74 |
| Σ *n*-6 LC-PUFA [§] | 2.36 | 0.09 | 10.60 [a] | 0.10 | 4.50 [b] | 0.09 | 4.00 [c] | 0.10 |
| Σ $C_{18}$ *n*-3 PUFA [‖] | 5.19 | 0.34 | 0.99 [c] | 0.05 | 41.21 [a] | 0.92 | 3.38 [b] | 0.08 |
| Σ *n*-3 LC-PUFA [¶] | 45.89 | 1.17 | 3.47 [c] | 0.03 | 17.21 [b] | 0.47 | 25.27 [a] | 0.79 |
| *n*-3/*n*-6 [**] | 6.75 | 0.11 | 0.12 [c] | 0.00 | 1.39 [a] | 0.00 | 1.05 [b] | 0.00 |
| *n*-3/*n*-6 LC-PUFA [††] | 19.44 | 0.22 | 0.33 [c] | 0.00 | 3.83 [b] | 0.04 | 6.31 [a] | 0.04 |

SO, sunflower oil-based diet; LO, linseed oil-based diet; FO, fish oil-based diet; LC-PUFA, long-chain PUFA (≥ 20C). [a,b,c] Mean values within a row with no common superscript letter were significantly different (Tukey's (parametric, $\alpha$ 5 %) or Wilcoxon's (nonparametric, $\alpha$ 1.69 %) *post hoc* tests on square root transformed final condition values).

[*] Sum of SFA, includes 20:0, 22:0 and 24:0.

[†] Sum of MUFA, includes 14:1n-5, 22:1n-9 and 24:1n-9.

[‡] Sum of *n*-6 PUFA with 18 C.

[§] Sum of *n*-6 long-chain PUFA with 20 C and 22 C.

[‖] Sum of *n*-3 PUFA with 18 C.

[¶] Sum of *n*-3 long-chain PUFA with 20 C and 22 C.

[**] Ratio of total *n*-3 PUFA to total *n*-6 PUFA.

[††] Ratio of *n*-3 long-chain PUFA to *n*-6 long-chain PUFA.

**Table 4.5.** Fatty acid composition (mg/g DM) of fish held on dietary treatments differing in dietary lipid source on the 10th (day 70) and the end (day 96) of the 36-day experimental period
(Mean values with standard error of the mean; *n* = 3 except SO/FO treatment at day 70 (*n* = 2))

| | Day 70 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SO | | LO | | FO | | SO/LO | | SO/FO | |
| Fatty acids | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 14:0 | 2.96 [c] | 0.08 | 2.32 [d] | 0.05 | 7.19 [a] | 0.08 | 2.77 [c] | 0.09 | 4.45 [b] | 0.00 |
| 16:0 | 30.22 [b] | 0.90 | 32.71 [b] | 1.38 | 40.46 [a] | 0.54 | 32.04 [b] | 1.31 | 33.13 [b] | 0.26 |
| 18:0 | 8.65 [b] | 0.26 | 10.78 [a] | 0.53 | 8.96 [b] | 0.16 | 9.68 [a,b] | 0.38 | 8.05 [b] | 0.01 |
| 16:1*n*-7 | 9.60 [b,c] | 0.43 | 9.37 [c] | 0.49 | 16.20 [a] | 0.26 | 9.39 [b,c] | 0.48 | 11.58 [b] | 0.08 |
| 18:1*n*-7 | 3.64 [c] | 0.16 | 3.34 [c] | 0.22 | 6.51 [a] | 0.09 | 3.61 [c] | 0.10 | 4.66 [b] | 0.02 |
| 18:1*n*-9 | 120.29 [a] | 4.53 | 58.29 [c] | 2.85 | 50.86 [c] | 0.80 | 98.20 [b] | 4.17 | 87.13 [b] | 0.50 |
| 20:1*n*-9 | 4.36 [c] | 0.14 | 2.11 [e] | 0.16 | 9.66 [a] | 0.17 | 3.57 [d] | 0.08 | 6.14 [b] | 0.03 |
| 18:2*n*-6 | 24.56 [c] | 0.81 | 33.88 [a] | 1.01 | 22.32 [c] | 0.31 | 29.14 [a,b] | 0.91 | 22.97 [c] | 0.13 |
| 18:3*n*-6 | 2.68 [a] | 0.16 | 1.27 [c] | 0.05 | 0.62 [d] | 0.01 | 2.18 [b] | 0.06 | 1.98 [b] | 0.07 |
| 20:2*n*-6 | 1.32 [c] | 0.04 | 1.71 [a] | 0.09 | 1.58 [a,b] | 0.00 | 1.49 [a,b,c] | 0.03 | 1.42 [b,c] | 0.05 |
| 20:3*n*-6 | 2.15 [a] | 0.17 | 1.66 [b] | 0.10 | 0.98 [c] | 0.02 | 2.07 [a,b] | 0.06 | 1.67 [a,b] | 0.03 |
| 20:4*n*-6 | 3.29 [a] | 0.10 | 0.96 [c] | 0.06 | 0.97 [c] | 0.02 | 2.57 [b] | 0.10 | 2.46 [b] | 0.07 |
| 22:4*n*-6 | 0.46 [a] | 0.01 | 0.09 [c] | 0.01 | 0.08 [c] | 0.00 | 0.33 [b] | 0.01 | 0.30 [b] | 0.01 |
| 22:5*n*-6 | 3.08 [a] | 0.07 | 0.65 [c] | 0.08 | 0.69 [c] | 0.05 | 2.22 [b] | 0.08 | 2.37 [b] | 0.14 |
| 18:3*n*-3 | 0.45 [d] | 0.07 | 38.37 [a] | 0.89 | 1.91 [c] | 0.03 | 15.94 [b] | 0.32 | 1.44 [c] | 0.17 |
| 18:4*n*-3 | 0.22 [e] | 0.01 | 4.94 [a] | 0.05 | 1.25 [c] | 0.01 | 1.99 [b] | 0.07 | 0.70 [d] | 0.03 |

**Table 4.5** - Continued

| | Day 70 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SO | | LO | | FO | | SO/LO | | SO/FO | |
| Fatty acids | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 20:3*n*-3 | 0.01 [e] | 0.00 | 1.84 [a] | 0.07 | 0.26 [c] | 0.01 | 0.76 [b] | 0.01 | 0.13 [d] | 0.00 |
| 20:4*n*-3 | 0.37 [c] | 0.11 | 2.27 [a] | 0.24 | 1.35 [b] | 0.04 | 1.15 [b] | 0.05 | 1.15 [b] | 0.22 |
| 20:5*n*-3 | 0.44 [d] | 0.01 | 2.07 [b] | 0.11 | 4.48 [a] | 0.09 | 1.17 [c] | 0.03 | 2.13 [b] | 0.02 |
| 22:5*n*-3 | 0.11 [d] | 0.01 | 0.86 [b] | 0.09 | 1.47 [a] | 0.04 | 0.44 [c] | 0.02 | 0.70 [b] | 0.01 |
| 22:6*n*-3 | 1.99 [d] | 0.05 | 9.01 [b] | 0.59 | 16.96 [a] | 0.45 | 5.57 [c] | 0.24 | 8.32 [b] | 0.01 |
| Total | 223.09 | 7.91 | 219.76 | 9.00 | 197.81 | 2.98 | 228.19 | 8.40 | 205.28 | 0.01 |
| Σ SFA [*] | 43.01 [b] | 1.28 | 46.32 [b] | 1.99 | 57.12 [a] | 0.77 | 45.44 [b] | 1.85 | 46.50 [b] | 0.27 |
| Σ MUFA [†] | 138.93 [a] | 5.26 | 73.86 [c] | 3.79 | 85.76 [c] | 1.30 | 115.72 [b] | 4.88 | 111.02 [b] | 0.65 |
| Σ $C_{18}$ *n*-6 PUFA [‡] | 27.25 [b] | 0.97 | 35.16 [a] | 1.05 | 22.94 [c] | 0.32 | 31.32 [a] | 0.95 | 24.95 [b,c] | 0.06 |
| Σ *n*-6 LC-PUFA [§] | 10.29 [a] | 0.40 | 5.07 [c] | 0.32 | 4.30 [c] | 0.06 | 8.68 [b] | 0.25 | 8.22 [b] | 0.02 |
| Σ $C_{18}$ *n*-3 PUFA [‖] | 0.68 [e] | 0.08 | 43.32 [a] | 0.94 | 3.17 [c] | 0.03 | 17.94 [b] | 0.36 | 2.14 [d] | 0.20 |
| Σ *n*-3 LC-PUFA [¶] | 2.93 [e] | 0.18 | 16.04 [b] | 1.08 | 24.53 [a] | 0.62 | 9.10 [d] | 0.25 | 12.44 [c] | 0.26 |
| *n*-3/*n*-6 [**] | 0.10 [e] | 0.00 | 1.48 [a] | 0.01 | 1.02 [b] | 0.01 | 0.68 [c] | 0.01 | 0.44 [d] | 0.02 |
| *n*-3/*n*-6 LC-PUFA [††] | 0.28 [e] | 0.02 | 3.16 [b] | 0.06 | 5.71 [a] | 0.11 | 1.05 [d] | 0.01 | 1.51 [c] | 0.04 |

**Table 4.5** - Continued

| | Day 96 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SO | | LO | | FO | | SO/LO | | SO/FO | |
| Fatty acids | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 14:0 | 2.53 $^{r}$ | 0.09 | 2.74 $^{r}$ | 0.06 | 7.53 $^{p}$ | 0.03 | 2.53 $^{r}$ | 0.06 | 6.52 $^{q}$ | 0.13 |
| 16:0 | 28.53 $^{s}$ | 0.53 | 39.06 $^{q}$ | 0.69 | 45.01 $^{p}$ | 0.25 | 35.19 $^{r}$ | 0.54 | 41.31 $^{q}$ | 0.64 |
| 18:0 | 8.99 $^{s}$ | 0.13 | 12.98 $^{p}$ | 0.24 | 9.96 $^{r}$ | 0.09 | 11.52 $^{q}$ | 0.17 | 9.26 $^{s}$ | 0.08 |
| 16:1*n*-7 | 8.31 $^{r}$ | 0.18 | 11.54 $^{q}$ | 0.43 | 17.92 $^{p}$ | 0.32 | 10.33 $^{q}$ | 0.47 | 15.91 $^{p}$ | 0.43 |
| 18:1*n*-7 | 3.57 $^{s}$ | 0.13 | 4.04 $^{r}$ | 0.11 | 7.52 $^{p}$ | 0.09 | 3.92 $^{r,s}$ | 0.06 | 6.67 $^{q}$ | 0.08 |
| 18:1*n*-9 | 118.16 $^{p}$ | 1.61 | 69.65 $^{r}$ | 1.22 | 57.23 $^{s}$ | 0.62 | 75.03 $^{q}$ | 0.17 | 65.71 $^{r}$ | 0.66 |
| 20:1*n*-9 | 4.60 $^{r}$ | 0.12 | 2.41 $^{s}$ | 0.09 | 10.88 $^{p}$ | 0.19 | 2.55 $^{s}$ | 0.06 | 9.55 $^{q}$ | 0.21 |
| 18:2*n*-6 | 24.43 $^{r}$ | 0.38 | 38.36 $^{p}$ | 0.42 | 24.68 $^{r}$ | 0.34 | 35.84 $^{q}$ | 0.39 | 24.75 $^{r}$ | 0.45 |
| 18:3*n*-6 | 2.39 $^{p}$ | 0.02 | 1.22 $^{r}$ | 0.01 | 0.47 $^{t}$ | 0.02 | 1.50 $^{q}$ | 0.04 | 0.85 $^{s}$ | 0.01 |
| 20:2*n*-6 | 1.39 $^{s}$ | 0.03 | 2.04 $^{p}$ | 0.01 | 1.83 $^{q,r}$ | 0.02 | 1.88 $^{q}$ | 0.02 | 1.74 $^{r}$ | 0.03 |
| 20:3*n*-6 | 2.15 $^{p}$ | 0.04 | 1.83 $^{q}$ | 0.05 | 1.02 $^{s}$ | 0.02 | 1.85 $^{q}$ | 0.02 | 1.25 $^{r}$ | 0.01 |
| 20:4*n*-6 | 3.42 $^{p}$ | 0.03 | 1.16 $^{r}$ | 0.02 | 1.13 $^{r}$ | 0.02 | 1.58 $^{q}$ | 0.01 | 1.51 $^{q}$ | 0.02 |
| 22:4*n*-6 | 0.46 $^{p}$ | 0.02 | 0.09 $^{r}$ | 0.01 | 0.10 $^{r}$ | 0.00 | 0.15 $^{q}$ | 0.01 | 0.16 $^{q}$ | 0.01 |
| 22:5*n*-6 | 3.60 $^{p}$ | 0.08 | 0.20 $^{s}$ | 0.01 | 0.33 $^{r}$ | 0.01 | 0.70 $^{q}$ | 0.04 | 0.83 $^{q}$ | 0.03 |
| 18:3*n*-3 | 0.51 $^{s}$ | 0.09 | 38.75 $^{p}$ | 1.04 | 2.05 $^{r}$ | 0.06 | 31.26 $^{q}$ | 1.07 | 1.85 $^{r}$ | 0.09 |
| 18:4*n*-3 | 0.20 $^{s}$ | 0.02 | 4.76 $^{p}$ | 0.04 | 1.34 $^{r}$ | 0.04 | 3.89 $^{q}$ | 0.17 | 1.13 $^{r}$ | 0.03 |
| 20:3*n*-3 | 0.01 $^{r}$ | 0.01 | 2.12 $^{p}$ | 0.08 | 0.33 $^{q}$ | 0.02 | 1.72 $^{p}$ | 0.08 | 0.28 $^{q}$ | 0.04 |

**Table 4.5** – Continued (day 96)

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 20:4*n*-3 | 0.08 $^{t}$ | 0.01 | 2.30 $^{p}$ | 0.07 | 1.36 $^{r}$ | 0.06 | 1.77 $^{q}$ | 0.01 | 1.04 $^{s}$ | 0.03 |
| 20:5*n*-3 | 0.26 $^{t}$ | 0.01 | 2.39 $^{r}$ | 0.03 | 5.22 $^{p}$ | 0.06 | 1.91 $^{s}$ | 0.03 | 4.40 $^{q}$ | 0.11 |
| 22:5*n*-3 | 0.05 $^{t}$ | 0.01 | 0.97 $^{r}$ | 0.03 | 1.72 $^{p}$ | 0.03 | 0.73 $^{s}$ | 0.02 | 1.43 $^{q}$ | 0.05 |
| 22:6*n*-3 | 1.44 $^{t}$ | 0.07 | 9.97 $^{r}$ | 0.17 | 19.01 $^{p}$ | 0.38 | 8.42 $^{s}$ | 0.17 | 16.22 $^{q}$ | 0.53 |
| Total | 217.47 $^{r}$ | 2.93 | 250.05 $^{p}$ | 3.28 | 220.29 $^{r}$ | 1.94 | 236.01 $^{q}$ | 2.51 | 215.80 $^{r}$ | 2.51 |
| Σ SFA $^{*}$ | 41.43 $^{s}$ | 0.76 | 55.42 $^{q}$ | 0.89 | 63.11 $^{p}$ | 0.37 | 50.09 $^{r}$ | 0.51 | 57.78 $^{q}$ | 0.83 |
| Σ MUFA $^{\dagger}$ | 135.63 $^{p}$ | 1.99 | 88.48 $^{s}$ | 1.51 | 96.58 $^{q,r}$ | 1.11 | 92.72 $^{r,s}$ | 0.42 | 100.59 $^{q}$ | 1.00 |
| Σ $C_{18}$ *n*-6 PUFA $^{\ddagger}$ | 26.81 $^{r}$ | 0.36 | 39.58 $^{p}$ | 0.43 | 25.15 $^{r}$ | 0.33 | 37.35 $^{q}$ | 0.42 | 25.60 $^{r}$ | 0.46 |
| Σ *n*-6 LC-PUFA $^{\S}$ | 11.02 $^{p}$ | 0.11 | 5.31 $^{r}$ | 0.03 | 4.41 $^{s}$ | 0.03 | 6.16 $^{q}$ | 0.07 | 5.49 $^{r}$ | 0.06 |
| Σ $C_{18}$ *n*-3 PUFA $^{\|}$ | 0.71 $^{s}$ | 0.10 | 43.51 $^{p}$ | 1.03 | 3.39 $^{r}$ | 0.06 | 35.15 $^{q}$ | 1.24 | 2.98 $^{r}$ | 0.07 |
| Σ *n*-3 LC-PUFA $^{\P}$ | 1.86 $^{t}$ | 0.10 | 17.75 $^{r}$ | 0.17 | 27.65 $^{p}$ | 0.52 | 14.54 $^{s}$ | 0.11 | 23.36 $^{q}$ | 0.64 |
| *n*-3/*n*-6 $^{**}$ | 0.07 $^{t}$ | 0.01 | 1.36 $^{p}$ | 0.01 | 1.05 $^{r}$ | 0.01 | 1.14 $^{q}$ | 0.02 | 0.85 $^{s}$ | 0.00 |
| *n*-3/*n*-6 LC-PUFA $^{\dagger\dagger}$ | 0.17 $^{t}$ | 0.01 | 3.34 $^{r}$ | 0.02 | 6.27 $^{p}$ | 0.11 | 2.36 $^{s}$ | 0.01 | 4.25 $^{q}$ | 0.08 |

SO, sunflower oil-based diet; LO, linseed oil-based diet; FO, fish oil-based diet; SO/LO, SO until day 60 and then LO from days 61 to 96; SO/FO, SO until day 60 and then FO from days 61 to 96; LC-PUFA, long-chain PUFA (≥ 20C). $^{a,b,c,d,e}$ for day 70 and $^{p,q,r,s,t}$ for day 96: mean values within a row with no common superscript letter were significantly different (Tukey's *post hoc* test on square root transformed values for each sampling day, $\alpha$ 5 %).

$^{*}$ Sum of SFA, includes 20:0, 22:0 and 24:0.

$^{\dagger}$ Sum of MUFA, includes 14:1n-5, 22:1n-9 and 24:1n-9.

$^{\ddagger}$ Sum of *n*-6 PUFA with 18 C.

$^{\S}$ Sum of *n*-6 long-chain PUFA with 20 C and 22 C.

$^{\|}$ Sum of *n*-3 PUFA with 18 C.

$^{\P}$ Sum of *n*-3 long-chain PUFA with 20 C and 22 C.

$^{**}$ Ratio of total *n*-3 PUFA to total *n*-6 PUFA.

$^{\dagger\dagger}$ Ratio of *n*-3 long-chain PUFA to *n*-6 long-chain PUFA.

### 4.3.3 *In vivo* fatty acid metabolism

Over the course of the entire feeding trial, total apparent *in vivo* SFA and MUFA elongation and Δ-9 desaturation activities were highest in fish subjected to the LO and FO treatments, while fish receiving SO recorded the highest total apparent *in vivo* SFA and MUFA β-oxidation (Tables 4.6 & 4.7). Within the *n*-6 PUFA family, fish fed on SO demonstrated a higher apparent *in vivo* elongation as well as higher Δ-5 and Δ-6 desaturation activities in comparison to those fed on LO and FO (Tables 4.6 & 4.7). In contrast, within the *n*-3 PUFA family, the highest apparent *in vivo* elongation, Δ-5 and Δ-6 desaturation activities were displayed in fish subjected to the LO treatment (Table 4.6). The apparent *in vivo* activities of fish at the end and on the $10^{th}$ day of the experimental period are reported in Tables 4.7 and 4.8, respectively. At the end of the experimental period, fish of the SO/LO group recorded lower apparent *in vivo* enzyme activities as compared to fish fed on LO at each elongation and desaturation step of the *n*-6 pathway. Similar observations were reported on the $10^{th}$ day of the experimental period, but only significantly for the apparent *in vivo* elongation activity (Table 4.8). In contrast, no differences in apparent *in vivo* elongation and desaturation activities within the *n*-3 pathway were observed between SO/LO and LO treatments, at the end of the trial or on the $10^{th}$ day of the experimental period (Tables 4.7 & 4.8). Considering both *n*-6 and *n*-3 pathways, similar apparent *in vivo* Δ-5 and Δ-6 desaturation activities were reported between SO/LO and LO fish groups. With respect to the dietary replacement of SO by FO (SO/FO), no statistical differences in apparent *in vivo n*-6 and *n*-3 PUFA enzyme activities were seen between SO/FO and FO fish groups on the $10^{th}$ day and at the end of the experimental period (Tables 4.7 & 4.8).

**Table 4.6.** Fatty acid metabolism (nmol/g per d), deduced by the whole body fatty acid balance method, of rainbow trout held on varying dietary lipid source diets for a 60-day pre-experimental feeding period
(Mean values with standard error of the mean; $n = 3$)

| | | SO | | LO | | FO | |
|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM |
| **SFA and MUFA** | **β-oxidation** | 223.5 [a] | 26.4 | 4.7 [b] | 0.8 | 26.0 [b] | 10.1 |
| | **Elongation** | 1865.3 [b] | 10.4 | 3850.3 [a] | 191.0 | 3205.0 [a] | 225.6 |
| | **Δ-9 desaturation** | 286.6 [c] | 3.6 | 921.1 [a] | 48.4 | 722.3 [b] | 49.0 |
| ***n*-6 PUFA** | **β-oxidation** | 173.0 [a] | 8.1 | 179.2 [a] | 26.0 | 4.8 [b] | 4.8 |
| | **Elongation** | 379.1 [a] | 5.4 | 111.6 [b] | 2.7 | 58.5 [c] | 2.6 |
| | 18:3*n*-6 to 20:3*n*-6 | 205.9 [a] | 1.7 | 66.0 [b] | 2.1 | 31.2 [c] | 0.7 |
| | 20:4*n*-6 to 22:4*n*-6 | 77.1 [a] | 1.6 | 5.4 [b] | 0.2 | 2.1 [c] | 0.4 |
| | 22:4*n*-6 to 24:4*n*-6 | 68.4 [a] | 1.4 | 4.2 [b] | 0.3 | 0.5 [c] | 0.2 |
| | **Δ-5 desaturation** | 153.1 [a] | 1.3 | 27.4 [b] | 0.9 | 10.2 [c] | 0.5 |
| | **Δ-6 desaturation** | 344.0 [a] | 2.0 | 92.2 [b] | 3.6 | 40.2 [c] | 0.9 |
| | 18:2*n*-6 to 18:3*n*-6 | 275.6 [a] | 1.0 | 88.0 [b] | 3.6 | 39.6 [c] | 0.7 |
| | 24:4*n*-6 to 24:5*n*-6 | 68.4 [a] | 1.4 | 4.2 [b] | 0.3 | 0.5 [c] | 0.2 |
| ***n*-3 PUFA** | **β-oxidation** | 38.4 [c] | 0.8 | 476.2 [a] | 41.1 | 198.9 [b] | 13.2 |
| | **Elongation** | 37.8 [c] | 2.0 | 761.9 [a] | 19.3 | 108.0 [b] | 22.4 |
| | 18:4*n*-3 to 20:4*n*-3 | 10.1 [b] | 1.1 | 309.7 [a] | 7.8 | 0.0 [c] | 0.0 |

**Table 4.6** - Continued

| | | SO | | LO | | FO | |
|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM |
| | 20:5*n*-3 to 22:5*n*-3 | 13.7 [c] | 0.5 | 215.7 [a] | 4.8 | 43.6 [b,c] | 11.8 |
| | 22:5*n*-3 to 24:5*n*-3 | 14.0 [c] | 0.4 | 200.6 [a] | 4.6 | 60.6 [b] | 11.0 |
| | **Δ-5 desaturation** | 11.0 [b] | 1.0 | 260.5 [a] | 5.8 | 0 [c] | 0 |
| | **Δ-6 desaturation** | 28.4 [c] | 1.3 | 637.9 [a] | 16.7 | 60.6 [b] | 11.0 |
| | 18:3*n*-3 to 18:4*n*-3 | 14.4 [b] | 1.0 | 437.3 [a] | 13.2 | 0.0 [c] | 0.0 |
| | 24:5*n*-3 to 24:6*n*-3 | 14.0 [c] | 0.4 | 200.6 [a] | 4.6 | 60.6 [b] | 11.0 |
| ***n*-6 & *n*-3 PUFA** | **Δ-5 desaturation** | 164.1 [b] | 2.2 | 287.9 [a] | 6.7 | 10.2 [c] | 0.5 |
| | **Δ-6 desaturation** | 372.5 [b] | 3.3 | 730.1 [a] | 20.2 | 100.8 [c] | 11.9 |

SO, sunflower oil-based diet; LO, linseed oil-based diet; FO, fish oil-based diet. [a,b,c] Mean values within a row with no common superscript letter were significantly different (Tukey's *post hoc* test on square root transformed values, $\alpha$ 5 %).

**Table 4.7.** Fatty acid metabolism (nmol/g per d), deduced by the whole body fatty acid balance method, of rainbow trout held on varying dietary lipid source diets for a 36-day experimental period after a 60-day pre-experimental period
(Mean values with standard error of the mean; $n = 3$)

| | | SO | | LO | | FO | | SO/LO | | SO/FO | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| **SFA and MUFA** | **β-oxidation** | 1545.9 [a] | 128.6 | 4.6 [c] | 0.8 | 10.3 [b] | 1.4 | 5.1 [c] | 0.8 | 12.5 [b] | 0.4 |
| | **Elongation** | 1006.4 [c] | 100.6 | 6824.6 [a] | 361.9 | 5580.6 [a,b] | 359.3 | 4889.1 [b] | 272.2 | 4520.6 [b] | 243.8 |
| | **Δ-9 desaturation** | 160.0 [c] | 7.9 | 1582.2 [a] | 85.3 | 1234.9 [b] | 88.9 | 1078.9 [b] | 67.2 | 944.9 [b] | 60.3 |
| ***n*-6 PUFA** | **β-oxidation** | 548.7 [a] | 26.4 | 159.3 [b] | 27.0 | 0.0 [c] | 0.0 | 212.1 [b] | 40.4 | 1.8 [c] | 0.3 |
| | **Elongation** | 348.9 [a] | 7.7 | 162.3 [b] | 2.9 | 82.9 [d] | 3.8 | 134.4 [c] | 4.4 | 78.7 [d] | 1.2 |
| | 18:3*n*-6 to 20:3*n*-6 | 180.3 [a] | 3.4 | 91.3 [b] | 2.4 | 40.3 [d] | 1.7 | 76.7 [c] | 2.7 | 36.4 [d] | 1.0 |
| | 20:4*n*-6 to 22:4*n*-6 | 74.7 [a] | 2.7 | 7.6 [b] | 0.4 | 3.4 [c] | 0.3 | 2.3 [c] | 0.7 | 2.6 [c] | 0.3 |
| | 22:4*n*-6 to 24:4*n*-6 | 66.6 [a] | 2.2 | 5.0 [b] | 0.2 | 0.7 [c] | 0.2 | 0.3 [c,d] | 0.3 | 0.0 [d] | 0.0 |
| | **Δ-5 desaturation** | 141.5 [a] | 3.0 | 41.3 [b] | 1.2 | 16.5 [d] | 0.4 | 31.2 [c] | 1.2 | 12.6 [e] | 0.2 |
| | **Δ-6 desaturation** | 293.1 [a] | 5.9 | 120.6 [b] | 4.3 | 52.4 [d] | 2.5 | 98.8 [c] | 4.8 | 43.5 [d] | 1.4 |
| | 18:2*n*-6 to 18:3*n*-6 | 226.4 [a] | 3.7 | 115.6 [b] | 4.1 | 51.7 [d] | 2.2 | 98.5 [c] | 4.6 | 43.5 [d] | 1.4 |
| | 24:4*n*-6 to 24:5*n*-6 | 66.6 [a] | 2.2 | 5.0 [b] | 0.2 | 0.7 [c] | 0.2 | 0.3 [c,d] | 0.3 | 0.0 [d] | 0.0 |
| ***n*-3 PUFA** | **β-oxidation** | 47.2 [c] | 4.5 | 485.3 [a] | 59.3 | 142.4 [b] | 15.7 | 496.2 [a] | 64.6 | 157.7 [b] | 4.4 |
| | **Elongation** | 40.5 [c] | 6.1 | 1002.7 [a] | 26.4 | 239.4 [b] | 25.4 | 1010.9 [a] | 19.1 | 258.1 [b] | 20.8 |
| | 18:4*n*-3 to 20:4*n*-3 | 15.1 [b] | 2.3 | 412.0 [a] | 9.4 | 3.1 [c] | 1.6 | 410.4 [a] | 7.2 | 0.0 [c] | 0.0 |
| | 20:5*n*-3 to 22:5*n*-3 | 12.4 [c] | 1.8 | 279.2 [a] | 7.9 | 110.9 [b] | 12.4 | 283.1 [a] | 6.0 | 119.9 [b] | 11.4 |

**Table 4.7** - Continued

| | | SO | | LO | | FO | | SO/LO | | SO/FO | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| | 22:5*n*-3 to 24:5*n*-3 | 12.6 $^{c}$ | 1.8 | 253.1 $^{a}$ | 7.5 | 123.0 $^{b}$ | 11.6 | 260.1 $^{a}$ | 6.5 | 134.6 $^{b}$ | 11.0 |
| | **Δ-5 desaturation** | 13.4 $^{b}$ | 2.1 | 346.5 $^{a}$ | 9.9 | 0.0 $^{c}$ | 0.0 | 347.4 $^{a}$ | 6.4 | 0.0 $^{c}$ | 0.0 |
| | **Δ-6 desaturation** | 30.2 $^{c}$ | 4.5 | 808.6 $^{a}$ | 19.3 | 123.0 $^{b}$ | 11.6 | 821.7 $^{a}$ | 17.2 | 134.6 $^{b}$ | 11.0 |
| | 18:3*n*-3 to 18:4*n*-3 | 17.6 $^{b}$ | 2.7 | 555.5 $^{a}$ | 12.1 | 0.0 $^{c}$ | 0.0 | 561.5 $^{a}$ | 12.2 | 0.0 $^{c}$ | 0.0 |
| | 24:5*n*-3 to 24:6*n*-3 | 12.6 $^{c}$ | 1.8 | 253.1 $^{a}$ | 7.5 | 123.0 $^{b}$ | 11.6 | 260.1 $^{a}$ | 6.5 | 134.6 $^{b}$ | 11.0 |
| ***n*-6 and *n*-3 PUFA** | **Δ-5 desaturation** | 154.9 $^{b}$ | 3.0 | 387.8 $^{a}$ | 10.8 | 16.5 $^{c}$ | 0.4 | 378.6 $^{a}$ | 7.2 | 12.6 $^{c}$ | 0.2 |
| | **Δ-6 desaturation** | 323.3 $^{b}$ | 5.7 | 929.2 $^{a}$ | 23.4 | 175.4 $^{c}$ | 12.3 | 920.5 $^{a}$ | 21.3 | 178.1 $^{c}$ | 10.3 |

SO, sunflower oil-based diet; LO, linseed oil-based diet; FO, fish oil-based diet; SO/LO, SO until day 60 and then LO from days 61 to 96; SO/FO, SO until day 60 and then FO from days 61 to 96. $^{a,b,c,d,e}$ Mean values within a row with no common superscript letter were significantly different (Tukey's *post hoc* test on square root transformed values, $\alpha$ 5 %).

**Table 4.8.** Fatty acid metabolism (nmol/g per d), deduced by the whole body fatty acid balance method, of rainbow trout held on varying dietary lipid source diets for a 10-day experimental period after a 60-day pre-experimental period
(Mean values with standard error of the mean; $n$ = 3 except SO/FO treatment ($n$ = 2))

| | | SO | | LO | | FO | | SO/LO | | SO/FO | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| **SFA and MUFA** | **β-oxidation** | 1101.5 | 421.5 | 20.6 | 7.0 | 63.0 | 29.9 | 355.2 | 333.1 | 685.1 | 99.4 |
| | **Elongation** | 1994.9 | 374.3 | 6025.3 | 1511.3 | 6072.1 | 355.9 | 3045.2 | 964.3 | 1702.3 | 220.8 |
| | **Δ-9 desaturation** | 273.5 $^{b}$ | 53.9 | 1354.2 $^{a}$ | 340.3 | 1281.6 $^{a}$ | 61.8 | 506.5 $^{a,b}$ | 185.0 | 192.9 $^{b}$ | 9.8 |
| ***n*-6 PUFA** | **β-oxidation** | 463.6 $^{a}$ | 111.2 | 401.2 $^{a}$ | 118.7 | 12.9 $^{b}$ | 12.9 | 454.2 $^{a}$ | 135.7 | 200.3 $^{a,b}$ | 43.0 |
| | **Elongation** | 372.1 $^{a}$ | 45.4 | 305.6 $^{a,b}$ | 42.0 | 180.0 $^{b,c}$ | 19.0 | 142.6 $^{c}$ | 23.4 | 107.3 $^{c}$ | 34.5 |
| | 18:3*n*-6 to 20:3*n*-6 | 201.4 $^{a}$ | 29.9 | 141.5 $^{a,b}$ | 21.3 | 70.4 $^{b,c}$ | 5.0 | 75.2 $^{b,c}$ | 16.4 | 28.0 $^{c}$ | 9.8 |
| | 20:4*n*-6 to 22:4*n*-6 | 73.2 $^{a}$ | 6.5 | 50.9 $^{a,b}$ | 7.9 | 35.6 $^{a,b}$ | 5.5 | 8.8 $^{c}$ | 3.2 | 20.8 $^{b,c}$ | 13.3 |
| | 22:4*n*-6 to 24:4*n*-6 | 61.2 | 5.4 | 46.2 | 7.3 | 32.9 | 4.9 | 4.0 | 2.5 | 17.8 | 14.1 |
| | **Δ-5 desaturation** | 157.6 $^{a}$ | 15.8 | 85.0 $^{a,b}$ | 10.6 | 44.4 $^{b,c}$ | 6.9 | 31.7 $^{b,c}$ | 11.8 | 18.9 $^{c}$ | 10.2 |
| | **Δ-6 desaturation** | 339.3 $^{a}$ | 51.3 | 225.7 $^{a,b}$ | 36.0 | 136.5 $^{b,c}$ | 10.4 | 96.6 $^{b,c}$ | 22.0 | 50.8 $^{c}$ | 33.3 |
| | 18:2*n*-6 to 18:3*n*-6 | 278.0 $^{a}$ | 45.8 | 179.5 $^{a,b}$ | 30.7 | 103.6 $^{b,c}$ | 5.6 | 92.7 $^{b,c}$ | 19.7 | 32.9 $^{c}$ | 19.2 |
| | 24:4*n*-6 to 24:5*n*-6 | 61.2 | 5.4 | 46.2 | 7.3 | 32.9 | 4.9 | 4.0 | 2.5 | 17.8 | 14.1 |
| ***n*-3 PUFA** | **β-oxidation** | 38.8 | 15.6 | 439.5 | 162.4 | 302.5 | 57.0 | 360.2 | 80.0 | 186.5 | 44.3 |
| | **Elongation** | 58.4 $^{b}$ | 30.3 | 979.4 $^{a}$ | 208.9 | 141.0 $^{b}$ | 62.3 | 1221.1 $^{a}$ | 63.9 | 252.2 $^{b}$ | 46.4 |
| | 18:4*n*-3 to 20:4*n*-3 | 38.4 $^{b}$ | 18.2 | 414.9 $^{a}$ | 95.3 | 22.4 $^{b}$ | 2.6 | 509.3 $^{a}$ | 22.4 | 55.2 $^{b}$ | 21.2 |
| | 20:5*n*-3 to 22:5*n*-3 | 9.2 | 6.4 | 262.7 | 60.7 | 53.2 | 27.1 | 338.8 | 21.8 | 86.6 | 13.8 |

**Table 4.8** - Continued

| | | SO | | LO | | FO | | SO/LO | | SO/LO | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| | 22:5*n*-3 to 24:5*n*-3 | 9.4 | 5.8 | 230.1 | 51.9 | 65.4 | 33.1 | 310.5 | 20.4 | 105.7 | 11.4 |
| | **Δ-5 desaturation** | 9.7 [b] | 8.1 | 323.4 [a] | 74.3 | 0.0 [b] | 0.0 | 409.7 [a] | 24.4 | 0.0 [b] | 0.0 |
| | **Δ-6 desaturation** | 46.5 [b] | 24.4 | 842.9 [a] | 166.3 | 65.4 [b] | 33.1 | 992.5 [a] | 46.0 | 123.8 [b] | 29.5 |
| | 18:3*n*-3 to 18:4*n*-3 | 37.1 [b] | 18.6 | 612.8 [a] | 114.5 | 0.0 [b] | 0.0 | 681.9 [a] | 26.4 | 18.1 [b] | 18.1 |
| | 24:5*n*-3 to 24:6*n*-3 | 9.4 | 5.8 | 230.1 | 51.9 | 65.4 | 33.1 | 310.5 | 20.4 | 105.7 | 11.4 |
| ***n*-6 and *n*-3 PUFA** | **Δ-5 desaturation** | 167.4 [b] | 21.5 | 408.4 [a] | 84.5 | 44.4 [c] | 6.9 | 441.4 [a] | 36.2 | 18.9 [c] | 10.2 |
| | **Δ-6 desaturation** | 385.8 [b] | 67.1 | 1068.6 [a] | 201.3 | 201.9 [b] | 42.3 | 1089.1 [a] | 67.6 | 174.6 [b] | 62.8 |

SO, sunflower oil-based diet; LO, linseed oil-based diet; FO, fish oil-based diet; SO/LO, SO until day 60 and then LO from days 61 to 96; SO/FO, SO until day 60 and then FO from days 61 to 96. [a,b,c,d,e] Mean values within a row with no common superscript letter were significantly different (Tukey's (parametric, $\alpha$ 5 %) or Wilcoxon's (nonparametric, $\alpha$ 1.69 %) *post hoc* tests on square root transformed values).

## 4.4 Discussion

The aim of the present study was to evaluate the fatty acid bioconversion capacity of rainbow trout fry previously depleted in *n*-3 PUFA over a 60-day pre-experimental period and subsequently reverted to a diet rich in ALA or rich in EPA and DHA, for a 36-day experimental period. As controls, three other fish groups received SO, LO and FO throughout the 96-day feeding trial.

### 4.4.1 Fish growth

A negative impact of SO was observed on fish growth performance in comparison to fish fed on LO or FO for 96 d. These results contrast with previous studies adding regular LA-rich sunflower oil or fish oil as only dietary lipid source in diets of Atlantic salmon[44] and rainbow trout[19] where no difference in fish growth and proximate composition between the two fish groups was reported. However, these studies were conducted on fish of a larger size and used fish meal as the dietary protein source, which undoubtedly provided *n*-3 LC-PUFA to the diet, up to a level that might potentially meet the requirements for these health promoting nutrients. The fatty acid requirements of rainbow trout are 1 % ALA, 1 % LA and/or 0.5 % *n*-3 LC-PUFA in their diet (DM)[5]. The present lower growth of SO-fed fish was certainly due to the deficiency in essential ALA and *n*-3 LC-PUFA, as well as to an interconnected reduced feed intake. In contrast with the present results on SO fish, and in accordance with previous studies[14; 18; 45; 46], feeding LO for 96 d had no impact on fish growth. The replacement of SO by LO or FO for the 36-day experimental period significantly improved the growth of fish initially fed on SO. Indeed, the FE were higher in SO/LO and SO/FO fish groups as compared to the SO fish group and were similar to those observed in LO and FO fish groups, respectively. The present results demonstrate the rapid capacity of rainbow trout to cope with a change in dietary source. Turchini *et al.*[47] previously reported enhanced growth, termed 'lipo-compensatory growth', of Murray cod fed a plant-derived oil diet and then a fish oil diet in comparison to fish fed a fish oil diet throughout. Similar observations were also reported in Atlantic salmon when shifted from rapeseed oil to a fish oil diet[48] and for red seabream fed a soybean oil diet for 3 months and then a fish oil diet for 32 d[27].

### 4.4.2 Fish fatty acid composition

At the end of the pre-experimental period (day 60), a high depletion in C18 *n*-3 PUFA and *n*-3 LC-PUFA was observed in fish of the SO treatment. This led to conclude on the efficiency of the pre-experimental period duration and in turn on the adequacy of the study design for evaluating the effects of the body *n*-3 PUFA depletion on the fish fatty acid bioconversion capacity. Interestingly, the *n*-3 PUFA depletion continued throughout the rest of the feeding trial for the SO treatment, following a decreasing exponential curve, as highlighted at days 70 and 96. Several studies[14; 19; 22; 23; 44] have previously reported that the fatty acid composition of fish reflects that of the dietary lipid source. Similarly, in the present study, fish fed on the 18:1*n*-9-rich SO were the richest in 18:1*n*-9, whereas fish fed on LO presented a high ALA concentration while those fed on FO had the largest EPA and DHA concentrations. Certain discrepancies with the dietary fatty acid profile were evident in fish samples. For example, despite an absence of dietary *n*-6 LC-PUFA, these fatty acids were the highest in fish of the SO treatment, pointing towards an active *in vivo* metabolism. This result contrasts with previously published work on Atlantic salmon fed a 100 % LA-rich sunflower oil diet, where an increased 20:2*n*-6 concentration and decreased 20:4*n*-6 concentration were reported in comparison to fish fed a fish oil diet [44]. However, the present result is in line with the results of a study on rainbow trout fed a 100 % LA-rich sunflower oil diet in comparison to fish oil and linseed oil diets[19]. In similar fashion, fish fed on LO in the present study recorded the highest concentrations of *n*-3 PUFA fatty acid intermediates (18:4*n*-3, 20:3*n*-3 and 20:4*n*-3), despite being absent from the diet. The same observation was previously reported in rainbow trout fed a linseed oil diet for 112 d as compared to fish fed sunflower or fish oil diets[19]. As previously observed by numerous studies, the present observations highlight, firstly, the relatively high capacity of rainbow trout to endogenously convert dietary LA and ALA into *n*-6 and *n*-3 LC-PUFA, respectively, and secondly, the modulation of the fish bioconversion capacity induced by the dietary lipid source[9; 14; 17; 19; 20; 22; 31; 49]. Indeed, more bioconverted products were reported along the *n*-6 pathway in fish fed on SO considering that LA was one of major fatty acids present as substrate and that dietary ALA was almost absent, as previously observed in European sea bass[31]. Conversely, more bioconverted products of the *n*-3 pathway were observed in fish fed on LO as LA was present to a lesser extent than ALA and also considering the initial affinity of enzymes towards the *n*-3 PUFA as

compared to the *n*-6 PUFA family[14; 15; 49]. A high recovery rate in *n*-3 PUFA was observed for SO/LO and SO/FO fish at the end of the 36-day experimental period. Indeed, SO/LO and SO/FO fish recovered a fatty acid profile with more than 80 % of the $C_{18}$ *n*-3 PUFA and *n*-3 LC-PUFA values observed in fish fed on LO and FO, respectively, for 96 d. Interestingly, the transfer of Atlantic salmon previously fed a rapeseed oil diet for 50 weeks to a fish oil diet for 20 weeks also restored their EPA and DHA concentrations to 80 % of the levels found in fish fed on a fish oil diet for 70 weeks[22]. In European sea bass, 70 % recovery in EPA and DHA was reported in the flesh of fish fed a 40 % fish oil/60 % plant-derived oil blend for 64 weeks and then a fish oil finishing diet for a further 20 weeks, in comparison with fish fed fish oil throughout[26]. However, two notable differences are apparent between both of these studies and the present one. Indeed, the results of the previous studies were based on fillet data from harvestable size fish whereas the present recovery rates are based on whole body fatty acid composition of fish from 20 g to 50 g. Besides the recovery rates of 80 % observed at the end of the experimental period, the recovery rates in *n*-3 LC-PUFA were also reported for the 10$^{th}$ day of the period and achieved about 50 % for both the SO/LO and SO/FO fish groups. This means that the recovery in *n*-3 LC-PUFA was higher during the 10 first d of the 36-day experimental period than during the 26 subsequent d that followed. This observation corresponds to the well-established dilution kinetics following a decreasing exponential curve[8; 23; 41; 50]. For example, this phenomenon was previously observed in Atlantic salmon fed a linseed oil diet for 40 weeks and then a fish oil diet for a further 24 weeks, where a DHA recovery rate of 83 % was observed by the end of the 24-week finishing period, while already reaching 79 % by the 16$^{th}$ week of the finishing period[23]. Interestingly, the DHA recovery rate was not slower and lower than that of the other *n*-3 LC-PUFA since the recovery rate values were similar on one hand on the short term (day 70) and on the other hand on the long term (day 96).

### 4.4.3 *In vivo* acid metabolism

The whole body fatty acid balance method clearly demonstrated the significantly increased apparent *in vivo* elongation and desaturation activities with regard to the *n*-3 biosynthesis pathway in fish fed on LO and the *n*-6 pathway in fish fed on SO. The high apparent *in vivo* bioconversion capacity of rainbow trout fed plant-based diets is well established[16; 19; 20] and is

confirmed in the present study. In fish fed on LO, 25 % of the consumed ALA was being bioconverted into higher homologues on day 60 of the experiment, while this value reached 27 % on day 96. In comparison, 27 % of consumed ALA was also bioconverted in fish subjected to the SO/LO treatment from day 61 through day 96. In contrast with the present results, a previous study reported that only 12 % of consumed ALA was bioconverted in rainbow trout with an initial mean weight of ~ 90 g fed a linseed oil diet for 72 d, with the majority either being accumulated (58 %) or oxidised (30 %)[20]. However, that study used, on one hand, fish with a bigger size than ours, and, on the other hand, diets formulated with 7 % of fish meal and therefore supplying fish with dietary EPA and DHA[20].

At the end of the experimental period, no differences in apparent *in vivo* enzyme activity were observed along the *n*-3 pathway between the SO/LO and LO treatments. Moreover, no effects were observed on the 10$^{th}$ day of the experimental period. This indicates that the high *n*-3 PUFA depletion obtained with the SO treatment did not increase the apparent *in vivo* bioconversion of *n*-3 PUFA during the experimental period when ALA-rich linseed oil was present. It thus appears that the fish fatty acid composition has no importance, in contrast to the dietary fatty acid input, on the capacity of fish to convert ALA into *n*-3 LC-PUFA. Interestingly, the present study reported a significant impact of the *n*-3 PUFA depletion on the *n*-6 PUFA bioconversion capacity of SO/LO fish. Indeed, reduced apparent *in vivo* elongation, as well as apparent *in vivo* Δ-5 and Δ-6 desaturation activities along the *n*-6 pathway were observed in fish of the SO/LO treatment in comparison to those of the LO treatment. These decreased activities related to the *n*-6 pathway should point out that, in the case of fish previously depleted in n-3 PUFA, elongases and desaturases neglect the conversion of LA into n-6 LC-PUFA in the case of an ALA supply. Nevertheless, this did not correspond to increased apparent *in vivo* elongation or desaturation activities on the *n*-3 pathway and suggests that the effects are not always entirely predictable. In line with the results observed at the end of the 36-day experimental period, at the 10$^{th}$ day sampling point, the activities on the *n*-6 biosynthesis pathway appeared somewhat reduced in the SO/LO fish in comparison to the LO fish group. Recent studies have investigated the impact of *n*-3 PUFA deprived diets on fish fatty acid metabolism and *n*-3 LC-PUFA deposition/ retention[19; 31; 32]. Francis *et al.*[19] reported a modulatory effect on *n*-3 LC-PUFA deposition in rainbow trout fed a classic LA-rich sunflower oil diet and then a fish oil diet. The authors reported that

the *n*-6 PUFA from the sunflower oil diet evoked a sparing of *n*-3 LC-PUFA from catabolism and resulted in higher *n*-3 LC-PUFA deposition in fish[19]. A similar sparing effect was also reported for sunshine bass (*Morone chrysops* × *M. saxatilis*) fed a SFA-rich diet for which limited effects of fish oil replacement were reported on fillet fatty acid composition[51; 52]. More precisely, sunshine bass fed a 50 % coconut oil diet and then a finishing fish oil diet recovered more effectively the *n*-3 LC-PUFA content observed for fish fed a fish oil diet throughout than fish fed three other diets formulated with 50 % grapeseed, linseed, or poultry oils for the grow-out period[52]. The authors concluded that dietary SFA appeared to be a preferential substrate for catabolism and induced an increased *n*-3 LC-PUFA deposition during the finishing period[52]. The present study reported no effect of the fish *n*-3 PUFA depletion on the apparent *in vivo* enzyme activity along the *n*-3 pathway in the SO/LO fish group, even on the 10$^{th}$ day of the experimental period. Further experiments should be set up to verify the absence of a transient metabolic adaptation in response to a previous shortage in dietary *n*-3 PUFA, for instance on the second or third d of the experimental period. The results of Hagar and Hazel[53] support the validity of this suggestion by reporting that in rainbow trout acclimated at either 5 or 20°C and then transferred to the opposite temperature, an increase in hepatic Δ-6 desaturase activity within the first three days of temperature transfer prior to reverting to baseline values on the 6$^{th}$ day was observed. The whole body fatty acid balance method is nevertheless unsuitable for such short experimental periods of a few days and other evaluation tools should thus be used, such as gene expression and enzyme activity measurements at the tissue or cellular level[31; 54; 55; 56; 57; 58]. These approaches should be implemented in further studies specifically focussing on tissues, such as liver and intestine, especially during the first days after dietary lipid replacement.

The present study is based on the *n*-3 PUFA depletion of fish with an initial mean weight of 0.7 g, which means fish that were previously fed on a standard diet for about five weeks. Complementary studies targeting the previously reported nutritional programming phenomenon[31; 32; 33] may be performed. In such studies, the *n*-3 PUFA depletion starts at a much earlier stage, such as at the alevin stage and low *n*-3 LC-PUFA diets are used as first feeding and during a short period. For example, a 3-week early exposure of rainbow trout swim-up fry to a diet formulated with rapeseed oil, palm oil and linseed oil improved fish growth, feed intake and FE when the diet was used again 7 months later[33]. The lipid bioconversion capacity

could also be improved by impacting broodstock. A recent study reported that feeding broodstock gilthead sea bream with linseed oil induced long-term effects on the juvenile progeny fed a plant-based diet, as demonstrated by increased fish growth, feed efficiency and Δ-6 desaturase gene expression, as compared to juveniles from broodstock fed a fish oil diet[59]. In the present study, it was potentially tougher to highlight a difference of apparent *in vivo* enzyme activity than with other fish species, as rainbow trout possesses a high lipid bioconversion capacity. A similar experiment performed on another species possessing a reduced basal lipid bioconversion capacity might more readily highlight the potential stimulation of a *n*-3 PUFA depletion on the fatty acid bioconversion capacity. As examples, two previous studies on European sea bass reported increased Δ-6 desaturase gene expression in juveniles fed a *n*-3 LC-PUFA deficient diet when previously fed a *n*-3 LC-PUFA deficient larval diet, as compared to groups fed rich *n*-3 LC-PUFA larval diets[31; 32]. In contrast, the lipid bioconversion capacity of common carp was not improved when fed a traditional cereal diet enriched with 1 % plant-derived oil for 180 d and then a linseed oil or fish oil finishing diet for 30 d[24].

## 4.5 Conclusion

The present study demonstrated that the initial high bioconversion capacity of rainbow trout to convert ALA into *n*-3 LC-PUFA was not modulated by a *n*-3 PUFA depletion of fish fatty acid composition through feeding for 60 d with a diet rich in sunflower oil. Indeed, the apparent *in vivo* enzyme activities related to that bioconversion remained stable along the *n*-3 fatty acid pathway. In contrast, the fish *n*-3 PUFA depletion negatively modulated the *n*-6 PUFA bioconversion capacity of fish in terms of reduced apparent *in vivo* elongation and desaturation enzyme activities, both on the 10$^{th}$ day and at the end of the 36-day experimental period. Further research on salmonids and other fish species is required to enhance the knowledge on fish fatty acid bioconversion metabolism and to improve fish bioconversion capacity through nutritional intervention strategies.

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## 4.7 Additional data

The digestibility results used to implement the whole body fatty acid balance method are presented in Table 4.9.

**Table 4.9.** Apparent digestibility coefficients (%) of fatty acids for each dietary treatment used to determine apparent *in vivo* fatty acid metabolism (Tables 4.6, 4.7 and 4.8)

| | Diet | | | | |
|---|---|---|---|---|---|
| Fatty acid | SO | LO | FO | SO/LO | SO/FO |
| 14:0 | 67 | 79 | 96 | 83 | 97 |
| 16:0 | 90 | 95 | 94 | 96 | 94 |
| 18:0 | 88 | 96 | 93 | 97 | 94 |
| 16:1n-7 | 69 | 79 | 97 | 83 | 97 |
| 18:1n-7 | 96 | 98 | 97 | 98 | 98 |
| 18:1n-9 | 95 | 98 | 97 | 99 | 98 |
| 20:1n-9 | 89 | 89 | 96 | 90 | 97 |
| 18:2n-6 | 95 | 97 | 95 | 98 | 96 |
| 18:3n-6 | 100 | 100 | 100 | 100 | 100 |
| 20:2n-6 | 100 | 79 | 96 | 81 | 96 |
| 20:3n-6 | 100 | 91 | 95 | 94 | 96 |
| 20:4n-6 | 100 | 100 | 97 | 100 | 98 |
| 22:4n-6 | 100 | 100 | 100 | 100 | 100 |
| 22:5n-6 | 100 | 100 | 100 | 100 | 100 |
| 18:3n-3 | 94 | 99 | 96 | 99 | 96 |
| 18:4n-3 | 100 | 100 | 98 | 100 | 99 |
| 20:3n-3 | 100 | 96 | 94 | 98 | 93 |
| 20:4n-3 | 100 | 100 | 98 | 100 | 99 |
| 20:5n-3 | 100 | 100 | 98 | 100 | 99 |
| 22:5n-3 | 100 | 100 | 98 | 100 | 99 |
| 22:6n-3 | 100 | 100 | 98 | 100 | 98 |

# Chapter 5

## Impact of lignans on the polyunsaturated fatty acid metabolic processing in a rainbow trout (*Oncorhynchus mykiss*) cell line

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*Submitted as a short communication to Aquaculture*

## Foreword

We observed in Chapter 4 that the depletion in n-3 PUFA of fish by feeding them on a sunflower oil diet did not improve their subsequent fatty acid bioconversion capacity when fed a linseed oil diet. Other feeding strategies to produce fish with a high content in n-3 LC-PUFA while reducing dietary fish oil are currently investigated. An interesting strategy could be the dietary inclusion of bioactive compounds, such as lignans. These compounds are found in large amounts in sesame seeds and linseed and also, even in reduced amounts, in their derived oils. It has previously been reported that the sesame seed lignans have a beneficial effect on fish fatty acid bioconversion capacity. In contrast, no study has been carried out on the impact of linseed lignans. In this context, the present chapter focused on the effects of sesame seed lignans, linseed lignans and enterolignans (potentially formed from dietary lignans through the gut microbiota) as potential enzymatic modulators of the fatty acid bioconversion capacity of rainbow trout liver cell line RTL-W1 enriched in ALA. The incubation of RTL-W1 cells with ALA and lignans or the enterolignan END at 19°C lasted 48 h. The ALA concentration in the culture medium in order to enrich the RTL-W1 cells in ALA was set at 50 µM, since a similar concentration has been previously used with this cell line without inducing cytotoxic effects (Ferain *et al*., 2016). The lignans and the enterolignan were supplemented to the culture medium at a 50 µM concentration. This concentration has been selected in order to facilitate the comparison with previous *in vitro* studies (Trattner *et al*., 2008; Schiller Vestergren *et al*., 2011). Even though a slight cytotoxicity (10 %) has been observed with one sesame seed lignan (SES) and the enterolignan END when used at 50 µM, we selected this concentration as a compromise since no cytotoxic effect was observed for the linseed lignans (SDG and SECO) and the second sesame seed lignan (EPI). Moreover, the effects of phenolic compounds are generally tested at concentrations close to 50 µM (from 20 to 100 µM). The present results have been submitted to Aquaculture journal. The gene expression results, which are not included in the article, are presented and discussed as additional results.

## Abstract

In the context of fish oil replacement by alternative plant-derived oils in the fish feed, the influence of lignans, which are phenolic compounds present at significant concentrations in some plant-derived oils, was investigated on the omega-3 (n-3) fatty acid metabolism of the rainbow trout liver cell line RTL-W1 enriched in alpha-linolenic acid (ALA, 18:3n-3). The major linseed oil lignans, namely secoisolariciresinol diglucoside (SDG) and secoisolariciresinol (SECO), as well as the enterolignan enterodiol (END) were evaluated. Moreover, the effects of sesame oil lignans, namely sesamin (SES) and episesamin (EPI), were also investigated and compared to those previously observed in *in vivo* and *in vitro* studies which reported favourable effects on the fatty acid bioconversion capacity of salmonids. Cells were incubated with 50 µM ALA and 50 µM of SES, EPI, SDG, SECO or END for 48 h at 19°C. The cells incubated with ALA had significantly higher amounts of 18:4n-3, 20:4n-3 and 20:5n-3 (eicosapentaenoic acid, EPA) as compared to control cells. The supplementation with SES decreased the 18:4n-3, 20:4n-3 and EPA contents, which indicates an inhibition of the desaturation and elongation steps involved in the fatty acid bioconversion pathway. As compared to SES supplementation, EPI supplementation showed a limited impact by only reducing 18:4n-3 and 20:4n-3. Similarly, END supplementation decreased 18:4n-3 and EPA, suggesting an inhibition of the desaturation capacity. Conversely, the supplementation of linseed lignans SDG and SECO had no impact on the fatty acid bioconversion capacity of RTL-W1 liver cells. In conclusion, the present results on the sesame seed lignans SES and EPI contradict the positive effects previously reported on fish lipid metabolism. In contrast, no influence of the linseed lignans SDG and SECO was observed. This suggests that the presence of lignans in linseed oil has no impact on the fish fatty acid bioconversion capacity when the oil is included to the diet.



## Keywords

RTL-W1 cell line - Fatty acid metabolism – Salmonids - Linseed lignans - Sesame seed lignans - Enterolignans

## Highlights of the manuscript

1. The impact of lignans on the fatty acid bioconversion in rainbow trout liver cells was evaluated through an *in vitro* approach with the rainbow trout liver cell line RTL-W1 enriched in ALA.
2. The sesame seed lignan sesamin and, to a lesser extent, episesamin and the enterolignan enterodiol decreased the n-3 fatty acid bioconversion capacity of the cells.
3. The linseed lignan secoisolariciresinol diglucoside and secoisolariciresinol had no effects on the n-3 fatty acid bioconversion capacity of the cells.

## 5.1 Introduction

Historically, aquaculture of high value species has relied on feed formulated with fish oil and fish meal, which are finite and limited marine resources (FAO, 2014). In order to reduce fish oil inclusion into fish feed, some plant-derived oils are considered as promising alternatives since they can replace the dietary fish oil without compromising fish growth performance (Turchini *et al.*, 2009). However, most plant-derived oils lack n-3 long chain (> C20) polyunsaturated fatty acids (LC-PUFA) and their inclusion in fish feed results in a reduced content of health-promoting n-3 LC-PUFA, namely eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), in fish tissues (Turchini *et al.*, 2009; Hixson, 2014). Unlike most plant-derived oils, linseed oil is particularly rich in alpha-linolenic acid (ALA, 18:3n-3) (NRC, 2011). ALA is the precursor of the bioconversion pathway alternately desaturating and elongating n-3 polyunsaturated fatty acids (PUFA) to produce n-3 LC-PUFA. These steps are catalysed by the endoplasmic reticulum membrane-bound Δ5 and Δ6 fatty acid desaturases (Δ5 Fads2 and Δ6 Fads2) and fatty acid elongases 2 and 5 (Elovl2 and Elovl5). A peroxisomal chain shortening is also involved (Tocher, 2003). The inclusion of ALA-rich linseed oil into fish feed induces increased desaturation and elongation enzymatic activities and therefore an increased fish n-3 LC-PUFA content, while not reaching the n-3 LC-PUFA content of fish fed a fish oil diet (Turchini *et al.*, 2009; Francis *et al.*, 2014).

Lignans are polyphenolic compounds found in high concentration in some plants (Landete, 2012). Some of them possess the capacity to affect the lipid metabolism of mammals and fish (Ashakumary *et al.*, 1999; Trattner *et al.*, 2008a; 2008b). Linseed is a major source of the plant lignan secoisolariciresinol (SECO), mostly found as secoisolariciresinol diglucoside (SDG) in the seeds. Sesame seeds (*Sesamum indicum*) also contain a large amount of the lignan sesamin (SES) (Landete, 2012). During the refining of sesame oil, SES is epimerised in episesamin (EPI) (Jin and Hattori, 2011). In the gastrointestinal tract of mammals, SECO, SDG, SES and EPI are transformed into enterolignans, namely enterodiol (END) and enterolactone (ENL) (Wang, 2002; Liu *et al.*, 2006; Jin and Hattori, 2011). Some studies previously reported that the inclusion of SES and/or EPI to the diet induced an increased β-oxidation of fatty acids in rodents (Ashakumary *et al.*, 1999; Kushiro *et al.*, 2002). In fish, these lignans have been shown to increase the DHA content in rainbow trout (*Oncorhynchus mykiss*) fed a plant-based diet

(Trattner *et al.*, 2008a). Moreover, the supplementation of SES and EPI to the ALA-enriched culture medium of Atlantic salmon (*Salmo salar* L.) hepatocytes increased the bioconversion of ALA into DHA (Trattner *et al.*, 2008b) and the amounts of the 18:4n-3 and 20:4n-3 bioconversion intermediates (Schiller Vestergren *et al.*, 2011). However, these results contrast with those reporting no major effects on the DHA content in tissues of salmonids fed diets formulated with plant-derived oils and SES (Schiller Vestergren *et al.*, 2012; 2013). Considering linseed lignans and enterolignans, neither *in vivo* nor *in vitro* studies have been up to now carried out on their potential impact on fish lipid metabolism.

Considering on one hand the context of fish oil replacement by alternative ALA-rich plant-derived oils in the fish feed and on the other hand the dietary request for EPA and DHA-rich fish, the present study evaluated the influence of several lignans on the bioconversion of ALA into n-3 LC-PUFA. More precisely, the effects of SES, EPI, SECO, SDG and END were investigated on the n-3 PUFA elongation and desaturation capacity of the RTL-W1 cell line (Rainbow Trout Liver-Waterloo 1) (Lee *et al.*, 1993) enriched in ALA.

## 5.2 Experimental procedures

### 5.2.1 Chemicals and reagents

SES (≥ 95 % purity, CAS 607-80-7) was obtained from Cayman (Ann Arbor, MI, USA), EPI (99.9 % purity, CAS 133-03-9) from Chromadex (Irvine, CA, USA) and SDG (99 % purity, CAS 158932-33-3) from PhytoLab (Vestenbergsgreuth, Germany). SECO (≥ 95 % purity, CAS 29388-59-8) and END (≥ 95 % purity, CAS 80226-00-2) were purchased from Sigma-Aldrich (St Louis, MO, USA). The foetal bovine serum (FBS) was obtained from PAA Laboratories (Pasching, Austria) and fatty acid free-bovine serum albumin from Sigma-Aldrich. ALA, the internal standard tridecanoic acid (13:0) and pure fatty acid methyl esters for fatty acid identification and quantification were obtained from Larodan (Solna, Sweden) and Nu-Check Prep (Elysian, MN, USA).

### 5.2.2 RTL-W1 cell culture

The RTL-W1 cell line, derived from the liver of a 4 year-old male rainbow trout (Lee *et al.*, 1993), was supplied by Prof Bony (INRA, France) and

thawed at passage 38. The cells were routinely grown in monolayer in 75 or 175 cm² flasks (Cellstar, Greiner Bio-One, Vilvoorde, Belgium) at 19°C, as previously described (Ferain *et al.*, 2016). The growth medium contained Leibovitz-15 (L15) medium, 5 % (*v/v*) FBS and 1 % (*v/v*) penicillin-streptomycin mix and was renewed every 48 or 72 h. About every 7 days, cells reaching 80 % of confluence were detached using 0.125 % trypsin in phosphate-buffered saline supplemented with 0.03 % ethylenediaminetetraacetic acid (PBS-EDTA). Trypan Blue was used as exclusion dye for cell counting.

### 5.2.3 Incubation of RTL-W1 cells with ALA and modulators

Since the RTL-W1 growth medium is deprived in the precursor of n-3 LC-PUFA biosynthesis pathway, namely ALA, the impact of modulators (lignans/enterolignan) on the n-3 PUFA bioconversion capacity of RTL-W1 cells was tested in the presence of ALA. Four lignans and one enterolignan were investigated as modulators: SES, EPI, SDG, SECO and END. Moreover, three conditions were implemented as control: the "CT" cells were incubated with a basic medium, the "CT ALA" cells with basic medium enriched in ALA and the "CT VS" cells with the basic medium enriched in ALA and with the lignan vehicle solvent (VS), namely dimethyl sulfoxide (DMSO).

For each incubation condition (3 controls and 5 modulators tested), four independent repetitions were performed with RTL-W1 cells between passages 53 and 56. At the beginning of each passage, cells were seeded in eight 175 $cm^2$ flasks in the presence of a basic medium containing L15, 2% (*v/v*) of FBS and 1% (*v/v*) of penicillin-streptomycin mix. Five days after the seeding, the basic medium was removed and cells were incubated in control or experimental medium for 48 h at 19°C. A 48-hour incubation was chosen as it was previously applied on Atlantic salmon hepatocytes supplemented in SES and EPI (Trattner *et al.*, 2008b; Schiller Vestergren *et al.*, 2011). The CT cells were incubated in a control medium containing L15 medium with no phenol red, 2% (*v/v*) FBS and 1% (*v/v*) penicillin-streptomycin mix. The CT ALA cells were incubated in the control medium enriched with 50 µM ALA. ALA was supplied as a complex with fatty acid free-bovine serum albumin (4:1 mol:mol) as previously described (Best *et al.*, 2006).The CT VS cells were incubated in the control medium enriched with 50 µM ALA and 0.15% (*v/v*) DMSO. The five experimental media were composed of the control medium enriched with 50 µM ALA, 0.15% (*v/v*) DMSO and 50 µM

of SES, EPI, SDG, SECO or END. The composition of the control and experimental media is summarised in Table 5.1. The stock solutions of modulators were dissolved in pure DMSO at a 33.3 mM concentration. At the end of the incubation, cells were collected using 0.0625% trypsin in PBS-EDTA.

**Table 5.1.** Comparison of the control and experimental media used for the incubation of RTL-W1 cells for 48 h at 19°C.

| | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Component | CT | CT ALA | CT VS | SES | EPI | SDG | SECO | END |
| Control medium | x | x | x | x | x | x | x | x |
| ALA 50 µM | | x | x | x | x | x | x | x |
| DMSO 0.15 % | | | x | x | x | x | x | x |
| Modulator 50 µM | | | | x | x | x | x | x |

CT: control, VS: vehicle solvent, SES: sesamin, EPI: episesamin, SDG: secoisolariciresinol diglucoside, SECO: secoisolariciresinol, END: enterodiol. The control medium included L15 medium with no phenol red, 2 % (*v/v*) FBS and 1 % (*v/v*) penicillin-streptomycin mix.

The cytotoxicity of ALA, DMSO and each of the modulators was tested with the CellTiter-Blue (Promega, Leiden, The Netherlands) and 5-carboxyfluorescein diacetate acetoxymethyl ester (Life Technologies, Carlsbad, CA, USA) assays, as previously described (Ferain *et al.*, 2016), to determine the influence of these chemicals on the metabolic activity and membrane integrity, respectively.

### 5.2.4 Fatty acid profile analysis

After two washing steps with L15 supplemented with fatty acid free-bovine serum albumin (10 mg/l), approximately 800 000 cells were collected from each condition for lipid extraction according to a method adapted from Bligh and Dyer (1959) using chloroform:methanol:water (2:2:1.8, *v:v:v*). Tridecanoic acid (13:0) was used as internal standard. The extracted fatty acids were converted into fatty acid methyl esters as previously described (Schneider *et al.*, 2012) and subsequently separated by gas chromatography (GC). The GC Trace (Thermo Scientific, Milan, Italy) was equipped with an RT2560 capillary column (100 m × 0.25 mm internal diameter, 0.2 µm film thickness) (Restek, Bellefonte, PA, USA), an automatic injector and a flame ionisation detector kept at a constant temperature of 255°C. The system used hydrogen as carrier gas at an operating pressure of 200 kPa. The oven

temperature programme was as follows: an initial temperature of 80°C, which progressively increased at 25°C/min up to 175°C, a holding temperature of 175°C during 25 min followed by an increase at 10°C/min up to 200°C, a holding temperature of 200°C during 20 min followed by an increase at 10°C/min up to 220°C, a holding temperature of 220°C during 5 min followed by an increase at 10°C/min up to 235°C and a holding temperature of 235°C for 15 min. Each peak was identified by comparison of retention times with those for pure methyl ester standards. Data processing was performed using the ChromQuest software 5.0 (Thermo Finnigan, Milan, Italy).

### 5.2.5 Statistical analyses

Data are presented as mean ± standard error of the mean of four biological replicates ($n$ = 4). The analysis of the variance (one-way ANOVA) and the Tukey *post hoc* test ($P < 0.05$) were used to determine significant differences between conditions. Prior to statistical analyses, data were transformed with square root to meet the assumptions for statistical methods. Statistical analyses were performed using the JMP software (JMP Pro 12, SAS, Cary, NC, USA).

## 5.3 Results

Cytotoxicity tests were firstly performed to determine the potential toxicity of ALA, DMSO, and the modulators during the 48-hour incubation (data not shown). The incubation with 50 µM ALA alone or with DMSO or 50 µM EPI, SDG or SECO dissolved in DMSO did not decrease the cell viability (as estimated by metabolic activity and membrane integrity) while the incubation with 50 µM ALA and 50 µM SES or END dissolved in DMSO slightly reduced the cell viability by 10%.

The fatty acid profiles of RTL-W1 cells incubated for 48 h in the three control conditions or with the five different modulators are expressed in nmol per million of incubated cells (Table 5.2). The most abundant saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) identified in control cells were, in decreasing amounts, 18:1n-9, 16:0 and 18:0. The amounts of these three fatty acids were not significantly affected by any of the incubation conditions. Similarly, the n-6 PUFA profile of cells was not impacted by the supplementation of ALA, DMSO and any modulator into the medium. Within CT cells, EPA, 22:5n-3 and DHA were the most

abundant n-3 PUFA. The addition of ALA to the medium resulted in higher amounts of ALA and of the n-3 PUFA bioconverted products 18:4n-3, 20:4n-3 and EPA in CT ALA cells. In contrast, the 22:5n-3 and DHA contents were not affected by ALA addition. The modulator vehicle solvent, DMSO, did not impact the fatty acid composition of cells, as compared to the CT ALA condition. While the addition of the linseed lignans SDG and SECO to the culture medium had no impact on the fatty acid composition of cells, the addition of the other modulators to the culture medium affected the fatty acid profile of cells differently. The addition of SES significantly decreased the n-3 bioconverted product content by reducing the 18:4n-3, 20:4n-3 and EPA amounts, as compared to CT VS cells. In the presence of EPI in the culture medium, a significantly lower amount of 18:4n-3 and 20:4n-3 was observed as compared to the content recovered in CT VS cells. With lignan addition, the reduction of 18:4n-3, 20:4n-3 and EPA contents were stronger with the inclusion of SES, as compared to that of EPI. The contents of 18:4n-3 and EPA were reduced in cells incubated with END as compared to cells of the CT VS condition.

**Table 5.2.** Fatty acid composition of RTL-W1 cells after a 48-hour exposure to 50 µM of plant lignans or enterodiol in the presence of 0.15 % (*v/v*) DMSO and 50 µM ALA. Results are expressed as nmol.$10^6$ $cells^{-1}$ (± SEM, *n* = 4).

| Fatty acid | CT | CT ALA | CT VS | SES | EPI | SDG | SECO | END |
|---|---|---|---|---|---|---|---|---|
| 16:0 | 9.83 ± 0.56 | 9.18 ± 0.74 | 10.27 ± 0.88 | 10.61 ± 0.48 | 10.47 ± 0.68 | 9.02 ± 0.26 | 9.51 ± 0.66 | 9.42 ± 0.47 |
| 18:0 | 9.40 ± 0.64 | 10.69 ± 0.89 | 11.08 ± 0.43 | 10.48 ± 0.52 | 11.11 ± 0.76 | 10.44 ± 0.21 | 10.81 ± 0.68 | 10.28 ± 0.33 |
| 18:1n-7 | 3.15 ± 0.25 | 2.80 ± 0.28 | 2.84 ± 0.09 | 2.92 ± 0.10 | 3.07 ± 0.24 | 2.79 ± 0.13 | 2.79 ± 0.15 | 2.74 ± 0.15 |
| 18:1n-9 | 24.35 ± 1.61 | 20.21 ± 1.98 | 21.31 ± 0.79 | 22.01 ± 0.73 | 23.25 ± 2.12 | 20.12 ± 0.67 | 20.52 ± 1.12 | 20.13 ± 0.86 |
| 18:2n-6 | 2.46 ± 0.13 | 2.21 ± 0.23 | 2.39 ± 0.06 | 2.60 ± 0.22 | 2.58 ± 0.22 | 2.23 ± 0.05 | 2.58 ± 0.37 | 2.38 ± 0.09 |
| 18:3n-6 | 0.13 ± 0.08 | 0.05 ± 0.05 | 0.00 ± 0.00 | 0.05 ± 0.05 | 0.06 ± 0.06 | 0.00 ± 0.00 | 0.06 ± 0.06 | 0.00 ± 0.00 |
| 20:3n-6 | 2.06 ± 0.12 | 1.67 ± 0.17 | 1.77 ± 0.07 | 1.86 ± 0.05 | 1.98 ± 0.19 | 1.68 ± 0.04 | 1.70 ± 0.07 | 1.76 ± 0.06 |
| 20:4n-6 | 3.62 ± 0.24 | 3.64 ± 0.39 | 3.82 ± 0.10 | 3.73 ± 0.13 | 4.06 ± 0.41 | 3.62 ± 0.08 | 3.65 ± 0.16 | 3.59 ± 0.10 |
| 18:3n-3 | 0.00 ± 0.00 $^{b}$ | 11.93 ± 1.17 $^{a}$ | 13.04 ± 0.24 $^{a}$ | 11.55 ± 0.51 $^{a}$ | 13.39 ± 1.00 $^{a}$ | 12.01 ± 0.22 $^{a}$ | 12.29 ± 0.73 $^{a}$ | 14.07 ± 0.77 $^{a}$ |
| 18:4n-3 | 0.00 ± 0.00 $^{e}$ | 5.38 ± 0.51 $^{a}$ | 5.42 ± 0.16 $^{a}$ | 1.93 ± 0.09 $^{d}$ | 4.09 ± 0.25 $^{bc}$ | 5.23 ± 0.12 $^{a}$ | 5.18 ± 0.17 $^{ab}$ | 4.00 ± 0.18 $^{c}$ |
| 20:3n-3 | 0.00 ± 0.00 | 0.20 ± 0.13 | 0.31 ± 0.12 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.22 ± 0.09 | 0.17 ± 0.11 | 0.40 ± 0.13 |
| 20:4n-3 | 0.00 ± 0.00 $^{c}$ | 0.86 ± 0.18 $^{ab}$ | 1.06 ± 0.07 $^{a}$ | 0.00 ± 0.00 $^{c}$ | 0.44 ± 0.17 $^{b}$ | 0.86 ± 0.04 $^{ab}$ | 0.67 ± 0.09 $^{ab}$ | 0.97 ± 0.03 $^{ab}$ |
| 20:5n-3 | 1.23 ± 0.08 $^{d}$ | 2.95 ± 0.31 $^{ab}$ | 3.14 ± 0.13 $^{a}$ | 1.67 ± 0.09 $^{cd}$ | 2.53 ± 0.21 $^{ab}$ | 2.87 ± 0.10 $^{ab}$ | 2.77 ± 0.13 $^{ab}$ | 2.22 ± 0.04 $^{bc}$ |
| 22:5n-3 | 1.96 ± 0.13 | 2.11 ± 0.22 | 2.30 ± 0.14 | 2.11 ± 0.06 | 2.31 ± 0.23 | 2.08 ± 0.05 | 2.08 ± 0.08 | 2.07 ± 0.07 |
| 22:6n-3 | 2.89 ± 0.18 | 2.90 ± 0.31 | 3.09 ± 0.10 | 3.06 ± 0.10 | 3.30 ± 0.33 | 2.91 ± 0.07 | 2.90 ± 0.13 | 2.90 ± 0.06 |

**Table 5.2** - Continued

| Fatty acid | CT | CT ALA | CT VS | SES | EPI | SDG | SECO | END |
|---|---|---|---|---|---|---|---|---|
| n-6 PUFA | 8.28 ± 0.51 | 7.59 ± 0.79 | 7.99 ± 0.22 | 8.24 ± 0.39 | 8.68 ± 0.83 | 7.53 ± 0.16 | 7.98 ± 0.59 | 7.73 ± 0.23 |
| n-3 PUFA | 6.09 ± 0.39 $^{c}$ | 26.33 ± 2.77 $^{ab}$ | 28.37 ± 0.63 $^{a}$ | 20.31 ± 0.78 $^{b}$ | 26.07 ± 1.77 $^{ab}$ | 26.19 ± 0.54 $^{ab}$ | 26.07 ± 1.29 $^{ab}$ | 26.63 ± 1.06 $^{a}$ |
| n-3 BC | 6.09 ± 0.39 $^{c}$ | 14.40 ± 1.60 $^{a}$ | 15.33 ± 0.47 $^{a}$ | 8.76 ± 0.32 $^{b}$ | 12.68 ± 0.77 $^{a}$ | 14.19 ± 0.44 $^{a}$ | 13.78 ± 0.58 $^{a}$ | 12.56 ± 0.35 $^{a}$ |
| Total | 61.79 ± 3.89 $^{b}$ | 76.8 ± 7.39 $^{ab}$ | 82.07 ± 30 $^{a}$ | 75.13 ± 2.80 $^{ab}$ | 83.12 ± 6.54 $^{a}$ | 76.25 ± 1.72 $^{ab}$ | 77.68 ± 4.33 $^{ab}$ | 77.15 ± 2.26 $^{ab}$ |

On the same line, mean values with no common superscript letter are significantly different ($P < 0.05$). CT: control, VS: vehicle solvent, SES: sesamin, EPI: episesamin, SDG: secoisolariciresinol diglucoside, SECO: secoisolariciresinol, END: enterodiol, n-6 PUFA: sum of 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6, n-3 PUFA: sum of 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 24:5n-3, 24:6n-3 and 22:6n-3, n-3 BC: sum of the bioconverted products 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 24:5n-3, 24:6n-3 and 22:6n-3, Total: sum of all identified fatty acids.

## 5.4 Discussion

The present study assessed and compared the capacity of five bioactive compounds to affect the bioconversion of ALA into more desaturated and elongated n-3 LC-PUFA in the RTL-W1 cell line. For this purpose, the influence on cellular fatty acid composition of four lignans, namely SES, EPI, SDG and SECO and of one enterolignan, namely END, were evaluated on cells enriched in ALA.

Untreated RTL-W1 cells (CT condition) were rich in SFA and MUFA, reflecting the fatty acid profile of the culture medium, in which FBS was the lipid source (Tocher *et al.*, 1988). The addition of ALA to the culture medium induced its uptake by the RTL-W1 cells and the production of the ALA-based Δ6 desaturation product, 18:4n-3, as well as its elongation product, 20:4n-3. Moreover, ALA supplementation also increased the amount of EPA in cells, resulted from the Δ5 desaturation of 20:4n-3. Previous studies already showed that the RTL-W1 (Ferain *et al.*, 2016), rainbow trout gonad (Tocher and Sargent, 1990), fathead minnow (*Pimephales promelas*, FHM) cell line (Gregory *et al.*, 2011), and Atlantic salmon (Ghioni *et al.*, 1999) cell lines were readily able to produce 18:4n-3, 20:4n-3 and EPA from ALA. Our results confirm the ability of RTL-W1 cells to convert ALA into n-3 LC-PUFA through Δ6 Fads2, Elovl5 and Δ5 Fads2 enzymatic activities. In contrast, the amount of the last products of the n-3 PUFA biosynthesis pathway, namely 22:5n-3 and DHA, did not increase with ALA enrichment. The low amount of 22:5n-3 in ALA enriched cells reflected a poor elongation rate of EPA into 22:5n-3. Since EPA is principally elongated by Elovl2 (Morais *et al.*, 2009; Gregory and James, 2014), the reduced elongation could be explained by a reduced Elovl2 enzymatic activity. The negligible synthesis of 22:5n-3 from ALA has already been observed in the RTL-W1 liver cells (Ferain *et al.*, 2016) but is in contradiction with results on the FHM cell line (Gregory *et al.*, 2011). In the present study, the addition of exogenous ALA to the culture medium did not modify the amount of DHA in CT ALA cells. In a previous study with RTL-W1 cells, even EPA supplementation (50 µM) did not induce the production of DHA (Ferain *et al.*, 2016), suggesting the loss of several enzymes including those of the peroxisomal chain shortening This observation is in line with the lack of peroxisomes in RTL-W1 cells observed by Malhao *et al.* (2013).

None of the modulators impacted the amount of SFA, MUFA and n-6 PUFA recovered in cells, as compared to the CT ALA and CT VS cells. In contrast, in isolated hepatocytes from Atlantic salmon, the addition of SES and EPI (50 µM) was reported to decrease the levels of 16:0, 18:1n-7 and 18:1n-9, expressed in relative percentages. The authors suggested an increased mitochondrial β-oxidation to explain these modifications (Trattner *et al.*, 2008b). In rodents fed SES and EPI, mechanisms involving the peroxisome proliferator-activated receptor alpha (Pparα) were suggested to be responsible for the increase of gene expression and activity of hepatic β-oxidation enzymes observed (Ashakumary *et al.*, 1999; Kushiro *et al.*, 2002). Moreover, the presence of the *pparα* gene has been demonstrated in the rainbow trout gill cell line (Liu *et al.*, 2005). The absence of effect observed in our experiments may therefore point out a different lignan impact on the Pparα system in RTL-W1 cells, as compared to Atlantic salmon hepatocytes in which a fatty acid β-oxidation increase was underlined (Trattner *et al.*, 2008b).

### 5.4.1 Modulation by SES and EPI

The positive effects of ALA enrichment on the fatty acid metabolism of cells were reduced by the SES supplementation. Thus, SES addition appeared to reduce the desaturation rates of ALA and 20:4n-3 and the elongation rate of 18:4n-3, leading to lower amounts of 18:4n-3, 20:4n-3 and EPA in SES exposed cells than in CT VS cells. Further experiments are required to conclude whether these reduced desaturations and elongation were due to an alteration of the Δ6 Fads2, Δ5 Fads2 and Elovl5 activities, or a down-regulation of the expression of their associated genes, namely fatty acid desaturase 2 (*fads2*) and elongases 2 and 5 (*elovl2* and *elovl5*), or a decrease of the *fads2*, *elovl2* and *elovl5* mRNA translation rates. The impact of SES on the ALA and 20:4n-3 desaturations may have also been mediated through the sterol regulatory element binding protein 1c (Srebp1c) and Pparα, as *fads2* expression is under the regulation of these transcription factors (Matsuzaka *et al.*, 2002; Nakamura and Nara, 2004). Previous studies (Ashakumary *et al.*, 1999; Ide *et al.*, 2001; Kushiro *et al.*, 2002) suggested that SES could act as Pparα ligand and also decrease the gene expression of *srebp1* and the formation of the Srebp1c mature form. As observed for SES, the addition of the lignan EPI also reduced the ALA desaturation rate and the 18:4n-3 elongation rate, leading to lower amounts of 18:4n-3 and 20:4n-3 in EPI exposed cells than in CT VS cells. While the high similarity between the

SES and EPI structures (Jin and Hattori, 2011) could explain similar mechanisms of action between the two modulators, the effects of SES addition were significantly more important as compared to the effects of EPI addition. Indeed, the amounts of 18:4n-3, 20:4n-3 and EPA were much lower in cells incubated with SES as compared to EPI. Differences between SES and EPI effects have previously been observed in the literature. For instance, in rats, EPI was more efficient than SES in increasing the hepatic fatty acid β-oxidation due to the higher EPI bioavailability in the liver (Kushiro *et al.*, 2002). Moreover, the incubation of Atlantic salmon isolated hepatocytes with a medium enriched in ALA and EPI up-regulated the *pparα* expression, unlike a SES and EPI equimolar-mixture supplementation, which appeared without effect on the *pparα* expression (Schiller Vestergren *et al.*, 2011). Conversely, the DNA synthesis inhibition in human leukaemia cells was more relevant with SES than EPI (Ju *et al.*, 2001). The different impact levels of SES and EPI observed in the present study may be explained by different absorption or catabolic rates, or a different efficiency to activate or bind to Pparα.

In contrast to our results, SES and EPI were found to stimulate the n-3 PUFA biosynthesis pathway in a primary culture of Atlantic salmon hepatocytes (Trattner *et al.*, 2008b; Schiller Vestergren *et al.*, 2011). Indeed, Trattner *et al.* (2008b) pointed out that the incubation of cells with an equimolar-mixture of SES and EPI (50 μM) during 48 h increased the bioconversion of ALA into DHA in a primary culture of Atlantic salmon hepatocytes. Similarly, Schiller Vestergren *et al.* (2011) demonstrated that the incubation of cells with an equimolar-mixture of SES and EPI or EPI alone (50 μM) increased the amounts of 18:4n-3 and 20:4n-3 in a primary culture of Atlantic salmon hepatocytes. The differences in the fatty acid composition of both cells and culture medium between the studies performed with primary cultures of hepatocytes and the present study on RTL-W1 cells may partially explain the contrasting results obtained. Indeed, Atlantic salmon were fed a fish oil-based commercial diet prior to the isolation of hepatocytes. As the lipid composition of isolated hepatocytes reflects the fish tissue of origin, the cells had higher EPA and DHA contents than the RTL-W1 cells, which were cultivated with 2% of FBS and had therefore a very limited access to n-3 LC-PUFA. Moreover, the culture medium used for both Atlantic salmon hepatocyte studies contained 7 μM of ALA whereas 50 μM of ALA were added to the culture media in the present study. In conclusion, the cells used in the present study had a fatty acid profile containing more

ALA and less n-3 LC-PUFA, as compared to the hepatocyte primary cultures. These differences in fatty acid composition have undoubtedly affected the membrane composition, which is known to modify the carrier-mediated transport and the activity of membrane-bound enzymes (Stubhaug *et al.*, 2005). A modified carrier-mediated transport may have altered the lignan cellular uptake and potentially lead to opposite effects if those are not linearly linked to the actual modulator intra-cellular concentrations. Similarly, a different activity of the desaturases and elongases, which are membrane-bound enzymes, may also explain the different effect of the sesame seed lignans on the ALA bioconversion. Considering that the expression of the *fads2* is under Srebp1c and Pparα regulations (Matsuzaka *et al.*, 2002; Nakamura and Nara, 2004), the high amount of DHA may have down-regulated the *fads2* gene expression through Srebp1c in the Atlantic salmon hepatocytes, as previously reported in a human liver cancer cell line (Xu *et al.*, 1999). The addition of SES and EPI, which are known to activate the Pparα system in rodents (Kushiro *et al.*, 2002), may have counteracted the down-regulation and may have therefore improved the bioconversion of ALA. Unlike n-3 LC-PUFA in the Atlantic salmon hepatocytes, the high amount of ALA may have up-regulated *fads2* gene expression through *pparα* in RTL-W1 cells, as it has been reported to be the case in rainbow trout liver slices (Coccia *et al.*, 2014). The addition of SES and EPI, which are known to inhibit the Srebp1c system (Ide *et al.*, 2001), may have therefore inhibited the up-regulation and may have thus reduced the bioconversion of ALA.

### 5.4.2 Modulation by SDG and SECO

The present study was the first one focusing on the impact of SDG and SECO on the n-3 fatty acid metabolism. SDG is the major lignan identified in linseed, which is the richest source of plant lignans (Landete, 2012). The present study demonstrated no apparent effect of linseed lignans on the lipid metabolism of RTL-W1 cells incubated with 50 µM ALA. This suggests that the presence of lignans, found in significant concentrations in linseed oil, is not a part of the explanation of the increased fatty acid bioconversion capacity observed in fish fed a linseed oil diet as compared to those fed a fish oil diet. Moreover, the present study suggests that linseed lignans have no detrimental effect on the fish fatty acid bioconversion capacity.

In humans and rats, SDG and SECO are known to reach the liver mainly as END and ENL since linseed lignans are rapidly metabolised by gastrointestinal bacteria before being absorbed by enterocytes (Wang, 2002;

Liu *et al.*, 2006). In fish, the transformation of SDG and SECO by gastrointestinal bacteria is unknown. Concerning the capacity of lignans to enter in RTL-W1 cells and therefore to induce a potential effect inside the cells, the lignan uptake by fish hepatocytes has never been investigated. Moreover, the absorption rate probably varies according to the lignan structure. Additional experiments are required to evaluate the actual uptake of these compounds by the RTL-W1 cells.

### 5.4.3 Modulation by END

As for linseed lignans, the present study is the first one to assess the impact of an enterolignan on the n-3 fatty acid metabolism in fish. In present study, the END supplementation induced a negative effect on the fatty acid metabolism regarding the decreased 18:4n-3 and EPA contents in cells as compared to the CT VS cells. Enterolignans are one of the major phytoestrogen classes, which can act as either oestrogen agonists or antagonists, depending on the biological level of oestradiol (Wang, 2002). In the human adipose tissue, oestrogens are known to down-regulate the expression of genes encoding the Δ5- and Δ9-desaturases (Lundholm *et al.*, 2008). END may have altered the RTL-W1 cell desaturation capacity through its oestrogenic activity. In humans and rats, both sesame seed and linseed lignans are considered as efficient enterolignan precursors (Peñalvo *et al.*, 2005; Liu *et al.*, 2006; Jin and Hattori, 2011). Therefore, the impact of END on the liver cells may reflect the *in vivo* influence of dietary SES, EPI, SDG and SECO on salmonid hepatic lipid metabolism. However, the existence of enterolignans in the salmonid gastrointestinal tract is still unknown.

## 5.5 Conclusion

The present study on the RTL-W1 cell line indicated that the ALA supplemented to the culture medium was readily absorbed and bioconverted into 18:4n-3, 20:4n-3 and EPA. The SES addition to the culture medium decreased the amount of 18:4n-3, 20:4n-3 and EPA, which indicates an inhibition of the desaturation and elongation steps involved in the transformation of ALA into EPA. EPI addition showed a limited impact as compared to SES addition, and only reduced the amount of 18:4n-3 and 20:4n-3 in cells. The negative impact of SES and EPI contrasts with the previous literature reporting a positive effect of sesame seed lignans on the fish fatty acid bioconversion capacity. The addition of the linseed lignans

SDG and SECO to the culture medium had no impact on the ALA bioconversion capacity of RTL-W1 cells. In a context of dietary fish oil replacement by ALA-rich plant-derived oils, the present results suggest that lignans of linseed oil are not involved in the high fatty acid bioconversion capacity observed in fish fed a linseed oil diet.

## 5.6 References

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## 5.7 Additional results

### 5.7.1 Experimental procedures

The extraction of total RNA was carried out using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. Briefly, the cell pellet (around 1 million cells) was thawed in the presence of the lysis solution (containing 1 % β-mercaptoethanol). Following addition of 70 % ethanol, the lysate was transferred into a RNA binding column and total RNA was extracted according to manufacturer's guidelines (DNAse treatment included). Total RNA was stored at -80°C after sampling of an aliquot for the RNA quantification and purity evaluation. The purity and quantity of the total RNA extracted were assessed with OD 260/280 and 260/230 using a ND-1000 NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). 1 µg of total RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad), as per kit instructions, and the cDNA samples were stored at -20°C until quantitative PCR experiments.

The relative expression of *fads2* and *elovl5-like* genes was measured by quantitative PCR (qPCR) on a Step One Plus PCR system from Applied Biosystems (Life Technologies). PCR primers were either designed according to rainbow trout cDNA sequences available in the European Nucleotide Archive database or sampled from the literature. For *fads2*, primers were obtained from Kamalam *et al.* (2013). For *elovl5-like* and *tubb2c*, primers were designed in consensus regions identified through alignments of corresponding transcript sequences available in the "data sheet 1" file of Marancik *et al.* (2015) (Table 5.3). Titration reactions were conducted to screen for optimal primer pair concentrations (100 nM for *elovl5-like*, 200 nM for *fads2*, 300 nM for *tubb2c*). Randomly selected amplicons (*i.e.*, full content of PCR microplate wells) were sent to Beckman Coulter Genomics for sequencing, as to verify sequence specificity. Expression of *tubb2c*, used as reference gene, was not significantly affected by experimental conditions (Table 5.3). The SYBR Select Master Mix (Life Technologies) was used in qPCR studies. The following conditions were applied: 2 min of initial denaturation at 95°C; 40 cycles of 15 s at 95°C then 1 min at 60°C; melt curve. Samples were analysed in triplicate. The relative expression of *fads2* and *elovl5-like* was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The SES condition and one biological

replicate had to be removed from the analysis to keep *tubb2c* as the reference gene.

**Table 5.3.** Primers used for gene expression determination by quantitative PCR.

| Gene | Primer | Sequence primer (5'-3') | T° annealing | Amplicon length |
|---|---|---|---|---|
| *tubb2c** | Forward | GGTCAATGTGGAAA TCAGATCG | 60 | 112 bp |
| | Reverse | TCTCTCAAGCTGCA GATCG | | |
| *fads2* | Forward | AGGGTGCCTCTGCT AACTGG | 60 | 175 bp |
| | Reverse | TGGTGTTGGTGATG GTAGGG | | |
| *elovl5-like* * | Forward | CCAAGTACATGAGA CACAGAC | 60 | 116 bp |
| | Reverse | CACACAGCAGACAC CATCTC | | |

*tubb2c, tubulin β 2c; fads2,* fatty acid desaturase 2; *elovl5-like,* fatty acid elongase 5. *Primers were designed based on consensus regions of several transcripts reported by Marancik *et al.* (2015). Transcripts can be found on European Nucleotide Archive, *tubb2c*: GSONMT00036168001, GSONMT00048685001, *elovl5*: GSONMT00036225001, GSONMT00065032001, GSONMT00071789001. *fads2* cDNA is reported in GenBank on the access number AF301910.

The analysis of the variance (one-way ANOVA) and the Tukey *post hoc* test ($P < 0.05$) were used to determine significant differences between conditions ($n = 3$). Data were first transformed with natural logarithm to meet the assumptions for statistical methods.

### 5.7.2 Results

The relative expression of *fads2* and *elovl5-like* genes are presented in Figure 5.1. No significant differences were observed between the seven incubation conditions for both genes.

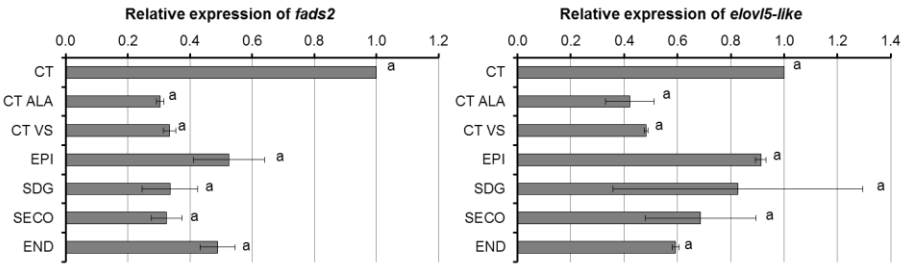


**Figure 5.1.** Relative gene expression of *fads2* and *elovl5-like* in RTL-W1 cells enriched in ALA with the 50 µM supplementation of plant or mammalian lignans to the culture medium for 48 h. Results are expressed as fold change (± SEM) from CT condition. On the same graph, data with no common letter are significantly different (Tukey's *post hoc* test, $P < 0.05$, $n = 3$). *fads2*, fatty acid desaturase 2; *elovl5-like*, fatty acid elongase 5; CT, control; VS, vehicle solvent; SES, sesamin; EPI, episesamin; SDG, secoisolariciresinol diglucoside; SECO, secoisolariciresinol; END, enterodiol.

### 5.7.3 Discussion

Even if no significant difference was observed, the relative expression of *fads2* and *elovl5-like* genes in RTL-W1 cells presented in Figure 5.1 should be interpreted with caution. Indeed, after the extraction of total RNA, the quantity and purity of the total RNA extracted were assessed (data not shown). The results display a low RNA concentration for few samples and a poor purity for some others; a low purity indicates the presence of unwanted organic compounds, which may influence the analysis. Moreover, the Ct values of the tubulin reference gene fluctuate in a range from 19 to 23 between the eight conditions (data not shown) even though tubulin is expected to be present with a stable expression between the conditions. The geometric mean of two or three reference genes is generally required for normalising the results. The elongation factor 1-α and β-actin reference genes were also tested (data not shown) but the important variation between the conditions rendered them unsuitable. In addition, a high variability was recorded between the results of the four biological replicates for some conditions. This variability may highlight various biological responses of RTL-W1 cells to a same treatment concerning the gene expression or may indicate technical problems. The SEM indicated on Figure 5.1 reflects these variations. Finally, no results of gene expression were obtained for the SES condition since tubulin expression was higher in this condition as compared to the seven other ones. This could reflect an effect of the SES

supplementation to the culture medium on the cytoskeleton of RTL-W1 cells.

In literature, the *pparα* and *fads2* gene expressions were reduced with the inclusion of a mixture of SES and EPI to the culture medium of Atlantic salmon hepatocytes (Trattner *et al.*, 2008b). Conversely, the incubation of Atlantic salmon hepatocytes enriched in ALA with SES or EPI increased the expression of *fads2* and *elovl5*, *elovl2* genes (Schiller Vestergren *et al.*, 2011). In a previous *in vivo* experiment, the dietary inclusion of SES and EPI to a plant-derived oil diet of rainbow trout induced a down-regulation of *pparα* (Trattner *et al.*, 2008a). This probably induced a down-regulation of *fads2* gene expression since *pparα* has a mediator role in the expression regulation of *fads2* (Matsuzaka *et al.*, 2002). In contrast, an up-regulation of *pparα* was reported in Atlantic salmon with the inclusion of SES and EPI to a plant-derived oil diet (Trattner *et al.*, 2011).

It is still therefore difficult to conclude on the impact of lignans on the expression of genes involved in the fatty acid bioconversion and present results cannot help to promote knowledge on this subject. The analysis of gene expression is nevertheless of great interest in research on potential effects of bioactive compounds on fish fatty acid bioconversion capacity.

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# Chapter 6

## General discussion and Perspectives

The present study aimed at evaluating the impact of temperature, fish n-3 PUFA depletion and several lignans on the fatty acid bioconversion capacity of the salmonid species rainbow trout in a context of global warming and of the unavoidable substitution of fish oil by alternative lipid sources in aquafeeds. More particularly, we focused our study on three items:

(i) the environmental impact of a 4°C-increase of the water temperature on the fatty acid bioconversion capacity of rainbow trout fed a plant-based diet,

(ii) the impact of a n-3 PUFA depletion of rainbow trout fry on its subsequent fatty acid bioconversion capacity when fed a n-3 PUFA rich diet,

(iii) the impact of several lignans as potential enzymatic modulators of the n-3 and n-6 PUFA biosynthesis pathways through an *in vitro* experiment with the rainbow trout liver cell line RTL-W1 enriched in ALA.

To achieve these goals, several experimental approaches have been implemented. The major outcomes derived from them, as well as some further considerations and perspectives, are given in the next paragraphs.

**An increase of water temperature induced negative effects on the fatty acid bioconversion capacity of rainbow trout fed a linseed oil diet.**

Climate change is known to affect oceans and freshwater ecosystems through several ways, such as increased water temperature induced by the global warming (Cochrane *et al.*, 2009; IPCC, 2014). By 2100, climate change will be associated with a gradual increase of surface temperature from 1 to 4°C (IPCC, 2014). Fish being ectothermic animals, the water temperature strongly influences their metabolism and physiology (Ficke *et al.*, 2007). Moreover, there is a need to find alternative lipid sources to fish oil currently present in significant amounts in aquafeeds of salmonids (Turchini *et al.*, 2009; Tocher, 2015). Plant-derived oils are one of the promising alternative lipid sources. It has already been reported that increased water temperature and plant-derived oil inclusion in fish feed affect the lipid metabolism of rainbow trout separately (Hagar and Hazel, 1985; Ingemansson *et al.*, 1993; Azevedo *et al.*, 1998; Vagner and Santigosa, 2011; Francis *et al.*, 2014) or simultaneously (Tocher *et al.*, 2004; Ng *et al.*, 2010; Wijekoon *et al.*, 2014). Nevertheless, to date, no study has investigated simultaneously the impacts of water temperature and dietary lipid source on rainbow trout lipid metabolism at both the whole fish and tissue levels, and with different complementary approaches.

In Chapter 3, we examined the concomitant impacts of increased water temperature and dietary fish oil replacement on the fatty acid bioconversion capacity of rainbow trout. For that purpose, fish fed diets formulated with fish oil or linseed oil (FO and LO diets, respectively) and were reared at the optimal water temperature of 15°C or the increased water temperature of 19°C for 60 feeding days. The effects were evaluated by analysing fish growth, whole fish, fillet and adipose tissue fatty acid compositions, apparent *in vivo* enzymatic activities of desaturases and elongases, hepatic and intestinal gene expression, and intestinal and hepatic Δ6 Fads2 activity.

The major outcomes considering the impact of increased water temperature on fish fed LO are as follows:

- Despite a higher feed intake, the fish growth was affected by the temperature increase as decreased FE was observed.
- The whole fish proximate composition was not affected by the temperature.

- The apparent *in vivo* activities of the Δ5 and Δ6 Fads2 were slightly reduced at the increased temperature of 19°C, whereas no impact was observed for elongases.
- The *fads2* expression was reduced in liver and intestine with the temperature increase.
- The microsomal Δ6 Fads2 activity measured in intestine was reduced with the temperature increase.
- The temperature increase slightly reduced the n-3 LC-PUFA content of fish. In contrast, a significantly increased ALA content was recorded.
- Whereas the fillet fatty acid composition was unaffected, the C18 n-3 PUFA and n-3 LC-PUFA contents of adipose tissue were increased with the temperature increase.

Overall, we observed that a temperature increase close to the upper limit of the species temperature tolerance range negatively affected the fatty acid metabolism of rainbow trout. This is consistent with the reduced hepatic elongase (*elovl2*) and *fads2* gene expression observed by Norambuena *et al.* (2015b) on Atlantic salmon fed diets varying in ARA/EPA ratio with a water temperature increase from 10°C to 20°C. Moreover, it has been reported that the Δ6 Fads2 activity was reduced in case of warm acclimation in freshwater fish (De Torrengo and Brenner, 1976; Hagar and Hazel, 1985; Tocher *et al.*, 2004). Interestingly, in the present study, the apparent *in vivo* activities of Δ5 and Δ6 Fads2 were reduced with the temperature but to a lesser extent in comparison with the results on gene expression and microsomal enzymatic activities. This highlights the differences that may appear when analysing environmental impacts at the whole body level or at the tissue level. At the whole body level, the relatively high fatty acid bioconversion capacity of rainbow trout, as compared to other fish species, might have minimized the impact of the temperature increase. Despite the temperature effects on fatty acid metabolism, the whole fish n-3 LC-PUFA content was only slightly affected by temperature and no temperature effect was reported on the fillet fatty acid composition. The unaltered n-3 LC-PUFA fillet content may be positive from a human nutrition perspective.

As reported in Section 1.4.3, a water temperature increase induces a reduced supply of dissolved oxygen. The oxygen solubility in fresh water is about 10 mg/l for a temperature of 15°C. With the temperature increase, the solubility decreases to about 9 mg/l at 20°C (Ficke *et al.*, 2007). Therefore, a reduced

supply in oxygen could be one of the explanations for the detrimental effects of increased temperature reported in the present work regarding the fish fatty acid metabolism. However, it is worth noting that the reduced oxygen supply cannot explain the reduced fish growth performance since the oxygen concentration was above 5 mg/l, which is required for an optimal growth for rainbow trout (Lucas and Southgate, 2012).

The present study was conducted at the water temperatures of either 15°C or 19°C, which are, respectively, the optimal growth temperature of rainbow trout and the upper limit of the species temperature tolerance range for growth (De Silva and Soto, 2009; Lucas and Southgate, 2012). However, in practical conditions, the water temperature varies daily and seasonally, and fish in their natural environment or in aquaculture are exposed to fluctuating water temperatures. In contrast, fish of the present study were exposed to stable temperatures during the feeding trial. A stable temperature is less physiologically demanding than naturally fluctuating water temperatures (Brett and Groves, 1979; Jalabert and Fostier, 2010). Therefore, the impact of climate change might induce more deleterious effects on fish growth and lipid metabolism considering that fish metabolic rates have to cope with daily/seasonal varying temperatures besides the water temperature increase induced by global warming. Interestingly, Morgan *et al.* (2001) observed that the growth of rainbow trout was improved in case of a 2°C temperature increase in winter whereas the opposite was observed in summer when temperatures were already high.

As highlighted by Cochrane *et al.* (2009), the climate change will influence the sustainability of the aquaculture development. As the water temperature impacts the physiology of fish, significant limitations for the aquaculture development emerge. For example, the efficiency of nutritional strategies such as the fish oil replacement with more sustainable lipid sources could be jeopardised by climatic changes. The salmonid farming is one of the aquaculture productions particularly threatened by climate change. Indeed, the fish meal and fish oil supplies from capture fisheries are expected to be negatively impacted by climate change. As the salmonid farming relies on fishmeal and fish oil inputs, such aquaculture systems are more threatened than other production systems (De Silva and Soto, 2009). Moreover, salmonids being cold-water species, their farming is expected to be more negatively affected as compared to the warm-water species farming. In the future, the salmonid farming could potentially become unsuitable for regions

traditionally rearing these species and production could move polewards or to cooler freshwaters, or be replaced with other fish species (Cochrane *et al.*, 2009). Some authors suggest the development of omnivorous and filter feeding fish farming as a measure against climate change effects (Naylor *et al.*, 2000; De Silva and Soto, 2009), as well as the research on salmonid strains tolerant to higher temperatures of 19°C to 20°C (De Silva and Soto, 2009).

Some perspectives result from the present study. The fatty acid composition of the fillet was unaltered by the water temperature, which is positive considering the n-3 LC-PUFA supply to human consumers. Nevertheless, this experiment was conducted on fish with a final weight of approximately 50 g. It would therefore be highly interesting to carry out a similar study on fish until a marketable size to conclude on the impact of increased water temperature on the n-3 LC-PUFA content of edible fish tissues.

The present study should be repeated in natural conditions of daily and seasonal water temperature variations through a longer period of one year in outdoor tanks. This would allow assessing the effects that farmed fish may face in a context of dietary fish oil replacement by plant-derived oils during their growth cycle in conditions more similar to those observed in salmonid farming.

The future experiments assessing the impact of water temperature increase should be designed considering the unavoidable replacement in fish oil by alternative dietary lipid sources. For instance, it has been reported that the complete replacement of fish oil by linseed oil or rapeseed oil affected the absorptive intestinal functions of rainbow trout and increased its intestinal permeability (Geurden *et al.*, 2009). The effect of the dietary lipid source on the intestinal integrity and permeability has to be taken in account in the evaluation of the susceptibility of fish to external stresses, such as the water temperature. For example, an increased risk of infection through increased translocation of bacteria may be caused by a disrupted epithelial barrier function associated to an increased water temperature (Geurden *et al.*, 2009). The concomitant impacts of increased temperature and dietary plant-derived oils should also be evaluated on the intestinal microbiota, which is notably required for efficient digestion and disease prevention. Indeed, the microbiota profile may change when plant-derived oils are included to the fish diet, as previously observed in rainbow trout (Ingerslev *et al.*, 2014). Moreover, it has been reported that the temperature was largely responsible

for the gut microbiota change observed in farmed Tasmanian Atlantic salmon (Neuman *et al.*, 2014). Interestingly, the influence on gut microbiota profile of fish can be easily studied by next-generation sequencing (NGS) (Ghanbari *et al.*, 2015).

In conclusion, the present study highlighted the negative impact that an increased water temperature could have on the fatty acid bioconversion capacity of rainbow trout. This effect is especially worrying in a context of fish oil replacement in aquafeeds, since the bioconversion capacity becomes then of major interest to convert the substrate of the n-3 PUFA bioconversion pathway, namely ALA, into its health-promoting n-3 LC-PUFA. Since the aquaculture is meant to provide the human consumer in n-3 LC-PUFA-rich fish, the development of fish feed production should take this unavoidable environmental issue into account.

**The replacement of fish oil by linseed oil in fish diet induced an increased apparent *in vivo* bioconversion capacity but decreased the n-3 LC-PUFA content of fish tissues.**

Another aspect developed in Chapter 3 was the dietary replacement of fish oil by linseed oil. No effect was reported on fish growth at the optimal water temperature of 15°C. At both temperatures, the dietary lipid source replacement induced a reduced n-3 LC-PUFA content in fillet and adipose tissue and increased LA and ALA contents. Higher apparent *in vivo* elongase, Δ5 and Δ6 desaturation activities were observed for fish fed LO. In contrast, the dietary treatment had no effect on *fads2* and *elovl5* expressions in liver and intestine. Moreover, no effect on the microsomal Δ6 Fads2 activity was observed.

Several studies previously reported that the dietary fish oil replacement by plant-derived oils did not compromise fish growth but reduced the n-3 LC-PUFA content in fish tissues (Bell *et al.*, 2004; Bell and Tocher, 2009; Turchini *et al.*, 2009; Turchini *et al.*, 2013a; Yildiz *et al.*, 2014). Despite we observed higher apparent *in vivo* desaturation and elongation activities in fish fed LO, as previously observed (Turchini and Francis, 2009; Thanuthong *et al.*, 2011c; Francis *et al.*, 2014), fish were not able to maintain a similar n-3 LC-PUFA content as fish fed FO. Considering gene expression results, the present ones are in contradiction with previous studies on freshwater fish reporting increased *fads2* and *elovl5* expressions in fish fed plant-derived oil diets (Seiliez *et al.*, 2001; Zheng *et al.*, 2005; Vagner

and Santigosa, 2011). The absence of effect on the Δ6 Fads2 activity is in accordance with results obtained in Eurasian perch fed fish oil or linseed oil diets (Geay *et al.*, 2015) but contrasts with most of the previous studies carried out on microsomes or isolated hepatocytes and reporting an increased activity for fish fed plant-derived oil diets (Buzzi *et al.*, 1996; Tocher *et al.*, 2003a; Vagner and Santigosa, 2011).

It appears from the results presented above that the dietary fish oil replacement still prompts research in order to achieve the development of feeding strategies allowing to sustain the n-3 LC-PUFA content of farmed fish fed n-3 LC-PUFA-deficient feeds. Indeed, we observed that the relative high fatty acid bioconversion capacity of rainbow trout is not sufficient in itself to maintain the n-3 LC-PUFA content reported for fish fed a marine fish oil diet. Therefore, some nutritional strategies are currently developed in order to increase the n-3 LC-PUFA content of fish fed plant-derived oil diets. The use of fish oil-based diets, termed finishing diets, distributed at the end of the grow-out period in order to restore the n-3 LC-PUFA content in fish previously fed plant-based diets has demonstrated positive results (Bell *et al.*, 2003; 2004; Mourente and Bell, 2006; Turchini *et al.*, 2011; Schultz *et al.*, 2015). In rainbow trout, finishing diets induced a shift in fish fatty acid profiles to a more fish oil-like composition, even though fish were unable to achieve similar n-3 LC-PUFA concentrations as compared to fish fed fish oil throughout their growth (Thanuthong *et al.*, 2012; Francis *et al.*, 2014). This feeding strategy allows reducing the fish oil input throughout the fish farming but still rely on fish oil for the last period before harvest.

The inclusion of n-3 LC-PUFA-rich alternative sources to fish oil is another interesting feeding strategy. For instance, algal oil could be the ideal replacement of fish oil (Hixson, 2014). Microalgae are the main primary producers of n-3 LC-PUFA and are currently used for the supply in EPA and DHA of rotifers (*Brachionus* spp.) and *Artemia nauplii*, which are life feed of some fish larvae (NRC, 2011; Tocher, 2015). However, in order to produce sufficient volumes of these microalgae to replace fish oil by algal oil and/or algal biomass as a source of n-3 LC-PUFA in fish feed, this alternative would imply high technological, biological and economic challenges (Miller *et al.*, 2010; Hixson, 2014). Nevertheless, the dinoflagellate microalgae and protists are currently used for the commercial production of DHA using large-scale biofermentors, but this costly production is currently intended to direct human consumption (Tocher,

2015). Some studies have reported promising results if microalgal oil is included to the feed of marine species at larval stages (Miller *et al.*, 2010). Interestingly, the dietary inclusion of dry algae meal has induced an increased n-3 LC-PUFA content in whole body of Atlantic salmon, without affecting growth and FE (Norambuena *et al.*, 2015a).

Considering the high potential of algae to biosynthesise n-3 LC-PUFA but the numerous issues associated to this production, another strategy to obtain n-3 LC-PUFA-rich sources could be the genetic transformation of oleaginous organisms with genes involved in the n-3 LC-PUFA production from microalgae. Currently, the only viable alternative to fish oil as n-3 LC-PUFA source is the metabolic engineering of oilseed crops with the capacity to synthesise n-3 LC-PUFA in seeds (Miller *et al.*, 2010; NRC, 2011; FAO, 2014; Hixson, 2014; Tocher, 2015). Ruiz-Lopez *et al.* (2014) reported that a transgenic *Camelina sativa* plant (initially rich in ALA) transformed with algal genes presented high contents in EPA, DHA or both in the seeds. The inclusion of transgenic *Camelina sativa* oil (richer in EPA or in EPA and DHA as compared to wild-type *Camelina sativa* oil) showed no impact on growth performance of Atlantic salmon (Betancor *et al.*, 2015; Betancor *et al.*, 2016). This highlights the feasibility of incorporation of such oils into the salmonid feed. However, the application of genetically modified ingredients in fish feed is notably challenged by the negative perception of consumers and the current lack of environmental evaluations (Shepherd *et al.*, 2016). An alternative approach has been used in Chile for the production of the so-called "Verlasso" salmon on the basis of the genetically modified oleaginous yeast *Yarrowia lipolytica* producing high amounts of EPA (Xue *et al.*, 2013). The yeasts are produced in biofermentors before to be added to the fish feed as dead cells in order to partially replace fish oil (Shepherd *et al.*, 2016). The use of oil from zooplankton, such as krill and copepods, as dietary n-3 LC-PUFA-rich source for farmed fish has also been suggested. However, the technological challenges, the cost and the environmental and ecological issues implied by such marine products limit their widespread use (NRC, 2011; Tocher, 2015).

In a perspective of increased n-3 LC-PUFA production by fish fed on plant-derived oils, the fish genetic engineering could be another option (Hixson, 2014; Tocher, 2015). Promising results have been obtained for zebrafish (Alimuddin *et al.*, 2005, 2007; 2008) and nibe croaker (*Nibea mitsukurii*) (Kabeya *et al.*, 2016). In zebrafish, the over-expression of an *elovl5-like*

gene from masu salmon (*Oncorhynchus masou*) induced higher EPA and DHA contents in fish (Alimuddin *et al.*, 2008). The authors also produced transgenic zebrafish by transformation with Δ5 and Δ6 desaturases of masu salmon (Alimuddin *et al.*, 2005, 2007). However, the increment in n-3 LC-PUFA was small and such strategy presents limitations based on the metabolic and physiological controls of the bioconversion pathway. Moreover, such genetic modifications are expected to face many sociological, ecological and environmental challenges (Tocher, 2015).

The n-3 LC-PUFA conservation in fish fed plant-derived oils could notably be reached through the enhancement of fish endogenous n-3 LC-PUFA production with the dietary inclusion of bioactive compounds, acting as enzymatic modulators of the n-3 LC-PUFA biosynthesis. The inclusion of micronutrients at specific levels (Senadheera *et al.*, 2012; Lewis *et al.*, 2013), 3-thia fatty acids (Kleveland *et al.*, 2006), CLA (Kennedy *et al.*, 2007) or lignans (Trattner *et al.*, 2008a; 2008b) have previously proved to be efficient in increasing the bioconversion capacity of salmonids, although to varying extents. Such bioactive compounds could be tested in feeding trials with rainbow trout at early stages of their development to specifically study their effects on the lipid bioconversion when at its highest capacity (Tocher *et al.*, 2003b; Bell and Dick, 2004).

An interesting mean to maintain a high level of n-3 LC-PUFA in fish while avoiding the use of dietary fish oil could be the concomitant dietary inclusions of (1) ALA-rich plant-derived oils to provide the substrate of the n-3 PUFA bioconversion pathway and therefore promote the endogenous production of n-3 LC-PUFA, (2) bioactive compounds to maximize the fish bioconversion capacity, and (3) alternative n-3 LC-PUFA-rich ingredients such as algae or transgenic plants. This last one might help compensating for the absence of n-3 LC-PUFA in conventional plant-derived oils, as well as for the limitations of the fish endogenous production and may respond to the human requirements.

On a methodological aspect, besides the Δ6 Fads2 enzymatic activity, the enzymatic activities of Δ5 Fads2 and elongases should be evaluated through a similar experiment and also with primary cultures to conclude on the impact of a temperature increase across the entire n-3 PUFA biosynthesis pathway. Moreover, the evaluation of enzymatic activities that we performed on intestinal samples should be repeated with liver samples since the liver is the major site of the desaturase and elongase activities.

In conclusion, additional research on an ideal feeding strategy is still required to produce farmed rainbow trout on a sustainable way while maintaining them a valuable source of n-3 LC-PUFA for human consumers. Since salmonids possess a high fatty acid bioconversion capacity as compared to other fish species, the optimisation of the n-3 LC-PUFA biosynthesis and retention is one of the approaches which have to be further developed, through nutritional strategies or genetic selection for instance. For that purpose, a thorough understanding of fish lipid metabolism and possible modulation of fish bioconversion capacity is needed.

**A significant n-3 PUFA depletion of rainbow trout fry does not modulate its subsequent fatty acid bioconversion capacity when fed a linseed oil diet.**

The inclusion of marine-derived fish oil in aquafeeds is widely criticised, from both economic and sustainable points of view (Tocher, 2015). In this context, numerous plant-derived oils have been evaluated as alternative dietary lipid sources but induced the production of farmed fish with a reduced n-3 LC-PUFA content (Bell and Tocher, 2009; Turchini *et al.*, 2009). This unavoidable fish oil replacement forces to develop feeding strategies to efficiently replace dietary fish oil and maintain the n-3 LC-PUFA level in fish at similar or higher levels than in farmed fish fed fish oil-based feeds (Turchini *et al.*, 2009; Tocher, 2015). The stimulation of the fatty acid bioconversion capacity of fish to increase the endogenous production of n-3 LC-PUFA from dietary ALA supply is one of these feeding strategies (Trattner *et al.*, 2008a; 2011; Giri *et al.*, 2016). The optimisation of the n-3 LC-PUFA gain and utilisation efficiency in fish tissues through nutritional conditioning during early larval stages is also a promising way (Vagner *et al.*, 2007; 2009). In the present work, we hypothesised that a severe n-3 PUFA depletion of fish could enhance its later fatty acid bioconversion capacity when fed a n-3 PUFA plant-based diet.

In Chapter 4, we evaluated the impact of a severe n-3 PUFA depletion imposed to rainbow trout fry on its subsequent fatty acid bioconversion capacity when fed an ALA-rich linseed oil diet. For that purpose, fish received a OLA-rich sunflower oil diet (SO) deficient in n-3 PUFA over a 60-day rearing period termed “pre-experimental period” and then a ALA-rich linseed oil diet (LO) or a EPA and DHA-rich fish oil diet (FO) over a 36-day rearing period termed “experimental period”, these treatments being called SO/LO and SO/FO, respectively. These treatments were compared to

fish continuously fed SO, LO or FO. Fish growth, fish fatty acid composition and apparent *in vivo* elongation and desaturation activities were evaluated at the end of both periods and on the 10$^{th}$ day of the experimental period.

The key results are the following:

- Growth performance was negatively affected for fish fed SO throughout the feeding trial whereas LO had no impact.
- The replacement of SO by LO (SO/LO) or FO (SO/FO) for the 36-day experimental period significantly improved the final fish weight, DGC and FE of fish initially fed SO.
- The pre-experimental period induced a severe depletion in n-3 PUFA for fish fed SO.
- SO/LO and SO/FO fish recovered a fatty acid profile with more than 80 % of the n-3 LC-PUFA content observed in fish fed on LO and FO throughout, respectively.
- As compared to fish fed FO, increased apparent *in vivo* elongation and desaturation activities were observed for fish fed plant-based diets with regard to the n-3 biosynthesis pathway in fish fed LO and the n-6 biosynthesis pathway in fish fed SO.
- At the end of the 36-day experimental period and on the 10$^{th}$ day, no difference in apparent *in vivo* enzymatic activity was observed along the n-3 biosynthesis pathway between the SO/LO and LO treatments.
- At the end of the 36-day experimental period, reduced apparent *in vivo* elongation, Δ5 and Δ6 desaturation activities along the n-6 biosynthesis pathway were observed in fish of the SO/LO treatment in comparison to those of the LO treatment.

As observed in Chapter 3 and as reported in the literature (Turchini and Francis, 2009; Francis *et al.*, 2014; Mellery *et al.*, 2016), the dietary inclusion of linseed oil induced an increase of the apparent *in vivo* desaturation and elongation enzymatic activities within the n-3 biosynthesis pathway. However, the pre-experimental n-3 PUFA depletion of fish did not stimulate the later apparent *in vivo* n-3 PUFA bioconversion capacity of trout fed the ALA-rich LO diet during the experimental period. In contrast, the reduced apparent *in vivo* bioconversion observed along the n-6 biosynthesis pathway reflects the negative modulation of the fish n-3 PUFA depletion within the n-6 PUFA family. These results should point out that, in the case

of an ALA supply, the desaturases and elongases neglect the bioconversions within the n-6 biosynthesis pathway at the expense of the n-3 pathway. Interestingly, despite the absence of stimulation of the bioconversion capacity for fish of the SO/LO treatment, the SO/LO fish recovered more than 80 % of the n-3 LC-PUFA observed in fish fed LO at the end of the 36-day experimental period. Such recovery rate has also been reported in Atlantic salmon fed a rapeseed oil diet and then a fish oil diet (Bell *et al.*, 2003). The consistency of results between the SO/LO and LO treatments may suggest that fish were performing at the upper limit of their bioconversion capacities. A sensible increase of the apparent *in vivo* desaturation activity on the $10^{th}$ day of the experimental period for SO/LO fish in comparison with LO fish could suggest a slight stimulation induced by the fish n-3 PUFA depletion, but this was not significant. Therefore, it is quite likely that even if a transient stimulation occurs, this could not help to significantly increase the n-3 LC-PUFA amount from endogenous production.

The apparent *in vivo* enzymatic activities were calculated via the implementation of the whole body fatty acid balance method, as developed and described by Turchini *et al.* (2007) and later modified (Turchini *et al.*, 2008; Turchini and Francis, 2009). It is interesting to note that the present study is the first one to implement the method on fish with an initial weight of 0.7 g. Indeed, previous studies using the method started at an initial fish weight of at least 5 g (Turchini *et al.*, 2013a; 2013b). Moreover, a low heterogeneity was reported within each treatment of our study, allowing highlighting significant differences with the statistical tests. Therefore, the present study allows concluding on the efficiency of the whole body fatty acid balance method conducted on feeding trials with a low initial fish weight.

As with all models, some limitations to the use of the whole body fatty acid balance method may simplify the actual biological processes. As reported by the authors who published the method, the calculations do not take into consideration the production of eicosanoids (Turchini *et al.*, 2007). This endogenous production could nevertheless be considered minor in comparison with the bioconversion, β-oxidation and direct storage and should have a limited impact on the total balance of fatty acids (Cunnane and Anderson, 1997; Francis *et al.*, 2007; Turchini *et al.*, 2007; Turchini and Francis, 2009). Another limitation is the assumption that a bioconverted fatty

acid is not further oxidised or chain-shortened. For example, if ALA is desaturated to 18:4n-3 and successively oxidised, it will be considered that it is ALA which is oxidised. However, it is reasonable to assume that the β-oxidation is predominant with LA and ALA, as compared to their more desaturated and elongated products, in fish fed SO or LO (Turchini *et al.*, 2007; Turchini and Francis, 2009). Finally, a sufficiently long period is required for the accurate assessment of the fish bioconversion capacity via the present method. Despite the limitations mentioned above, the method still offers a simple and reliable approach to estimate the overall fatty acid bioconversion capacity of fish within the context of an integrated system. Moreover, the listed limitations are also encountered with other methods employing labelled fatty acids (Turchini and Francis, 2009).

In a context of fish oil replacement by alternative lipid sources, sunflower oil appears to be an unsuitable candidate considering salmonid farming. In the present study and as previously observed (Turchini *et al.*, 2013a; Francis *et al.*, 2014), the apparent *in vivo* bioconversion was stimulated within the n-6 PUFA family in case of sunflower oil supply, this oil being rich in LA. This stimulation will not improve the n-3 LC-PUFA content of farmed fish. Moreover, a negative growth was observed for fish fed SO. This contrasts with studies on salmonids in which the dietary replacement of fish oil by sunflower oil had no impact on fish growth. It is however important to notice that these studies used fish meal as dietary protein source (Bransden *et al.*, 2003; Francis *et al.*, 2014). Fish meal is expected to provide the experimental fish with some n-3 LC-PUFA, among other nutrients. However, in an optic of sustainable aquafeed production, an effective fish growth could be requested with feeds that are totally based on plant-derived ingredients. In contrast to the sunflower oil, the ALA-rich linseed oil could be a more appropriate candidate to respond to the fish oil substitution challenge as it has been prove in the present study and in the literature (Bell *et al.*, 2004; Turchini and Francis, 2009; Thanuthong *et al.*, 2011c; Francis *et al.*, 2014; Mellery *et al.*, 2016) that this oil did not compromise fish growth and increased the endogenous production of n-3 LC-PUFA in salmonids. However, the linseed oil production represents a substantial trade, considering the seed as well as the oil (Gunstone, 2011), and other ALA-rich sources should also be used in order to alleviate the pressure on linseed oil demand for fish feed, human consumption and non-food industry. Other plant-derived oils rich in ALA exist and have been tested as dietary lipid source in fish diets. In Atlantic salmon and rainbow trout, the inclusion of

ALA-rich *Camelina sativa* oil as the main lipid source in diets did not affect the fish growth and increased the fish apparent *in vivo* fatty acid bioconversion (Hixson *et al.*, 2014). In both species, the authors reported that about 25 % of the dietary ALA supply was bioconverted into more desaturated and elongated fatty acids (Hixson *et al.*, 2014). The echium oil is also interesting considering its high contents in ALA and 18:4n-3 (Glencross, 2009). In Atlantic salmon, the complete dietary replacement of fish oil by echium oil had no impact on fish growth and FE and similar n-3 LC-PUFA amounts were recovered in fish fed diets based on fish oil or echium oil and reared from approximately 40 g to 80 g per fish (Miller *et al.*, 2007). Such plant-derived oils are promising alternatives to linseed oil as ALA-rich dietary lipid sources for inclusion in fish feed. However, for some of them, the feasibility of a large scale production has still to be considered.

Even if no effect of the n-3 PUFA depletion of fish was observed on its bioconversion capacity in the present study, it cannot be ruled out that a longer and/or a sooner n-3 PUFA depletion would have shown a dietary effect on fish metabolism. These conditions should thus be tested. As an example, an n-3 PUFA deficient diet could be used as first feeding and during a short period corresponding to a classical nutritional programming approach (Vagner *et al.*, 2007; Geurden *et al.*, 2009). Fish subjected to a n-3 PUFA deficient diet at the alevin stage, or through manipulation of the diet of the broodstock, have been suggested to accept a plant-derived diet at a higher level, to be less sensitive to hepatic stress (Geurden *et al.*, 2013; Balasubramanian *et al.*, 2016) and to be positively affected regarding the lipid metabolism. Interestingly, Geurden *et al.* (2013) reported that an early short-term exposure of rainbow trout fry to a plant-derived diet improved the feed intake, the FE and the fish growth when the same diet was given again at later life stages.

In order to stimulate the fatty acid bioconversion capacity, fish could be subjected to a similar experiment to the one reported in the present thesis with the additional input of bioactive compounds to the diet when fish are supplied in n-3 PUFA. Several bioactive compounds have been described above. Among them, some lignans have been tested in an *in vitro* approach in Chapter 5. The concomitant effect of the fish n-3 PUFA depletion and the presence of dietary bioactive compounds could lead to a better modulation of the n-3 and n-6 PUFA bioconversion pathways. Moreover, the sensible increase of apparent *in vivo* enzymatic activity observed during the first days

of the experimental period may be extended for a longer period of time thanks to the inclusion of bioactive compounds.

We assessed the apparent *in vivo* metabolism at 10 and 36 days after the dietary change of the n-3 PUFA deficient diet to the n-3 PUFA supply diet, and no effect was observed between SO/LO and LO. To go further ahead in the study, the fatty acid bioconversion capacity could be evaluated in the first days after the dietary change, for instance after two or three days. The *in vivo* whole body fatty acid balance method is nevertheless unsuitable to assess the bioconversion capacity in such a short period of time. Therefore, an *in vitro* approach could be implemented by the evaluation of the desaturase and elongase gene expressions in fish tissues and of the desaturase and elongase enzymatic activities in isolated cells or tissue microsomes with the inclusion of radio- or stable isotope-labelled fatty acids (Brown, 2005).

In conclusion, the present study demonstrated that the basal high bioconversion capacity of rainbow trout to convert ALA into n-3 LC-PUFA was not modulated by a severe n-3 PUFA depletion. Some perspectives have been proposed to modulate at a higher level or on a longer period the fatty acid bioconversion capacity of fish. However, it is reasonable to suppose that an increase of the bioconversion is not unlimited and if occurring throughout some days or weeks, it cannot be sufficient in itself to achieve the n-3 LC-PUFA content reported for fish fed a fish oil diet.

**The sesame seed lignan SES and, to a lesser extent EPI and the enterolignan END, reduced the fatty acid bioconversion of the RTL-W1 cell line whereas no effect of the linseed lignans SDG and SECO was reported.**

As reported in Chapters 3 and 4, the substitution of dietary fish oil by linseed oil induced a reduction of the n-3 LC-PUFA content in fish, despite the increased apparent *in vivo* fatty acid bioconversion capacity observed. This reduction has been widely observed in the literature when fish oil is replaced by plant-derived oils (Turchini *et al.*, 2009; Tocher, 2015). An interesting approach to increase n-3 LC-PUFA in fish fed a plant-derived oil diet could be the dietary inclusion of bioactive compounds in order to stimulate the fatty acid bioconversion. Lignans and enterolignans could be such compounds. Indeed, SES and EPI, which are lignans found at high concentrations in sesame seed, have shown interesting results in *in vivo*

studies when included to the fish diet (Trattner *et al.*, 2008a; Trattner *et al.*, 2011; Alhazzaa *et al.*, 2012), or in *in vitro* studies (Trattner *et al.*, 2008b; Schiller Vestergren *et al.*, 2011) (for detailed information see Section 1.3.3.2.2). Linseed is another rich source of lignans, namely SDG and SECO. Such lignans may enhance the fatty acid bioconversion capacity of fish fed a linseed oil diet. However, to date, no study has been carried out on the impact of other lignans or enterolignans as modulators of the lipid metabolism in fish.

Chapter 5 aimed at evaluating the impact of different lignans and enterolignans on the fatty acid bioconversion in the RTL-W1 cell line, which was selected in order to obtain fast and reproducible results for a relevant comparison. For that purpose, cells were enriched in ALA and one lignan (SES, EPI, SDG or SECO) or enterolignan (END) was added to the culture medium. Cells were afterwards incubated for 48 h at 19°C. Moreover, three conditions were implemented as control: the CT cells were incubated with the basic culture medium, the CT ALA cells with basic medium enriched in ALA and the CT VS cells with basic medium enriched in ALA and the lignan vehicle solvent DMSO. The bioconversion was evaluated by analysing the cellular fatty acid composition. The main results are the following:

- The bioconversion of ALA was effective in RTL-W1 cells as increased 18:4n-3, 20:3n-3, 20:4n-3 and EPA concentrations were reported in CT ALA cells. The bioconversion was nevertheless stopped from 22:5n-3.
- The SES inclusion to the culture medium decreased the 18:4n-3, 20:4n-3 and EPA concentrations in cells as compared to CT VS cells.
- EPI showed a more limited impact in comparison to SES by only reducing 18:4n-3 and 20:4n-3 in cells as compared to the CT VS cells.
- SDG and SECO demonstrated no apparent effect on the fatty acid bioconversion capacity of cells.
- The addition of END induced a decrease of the 18:4n-3 and EPA concentrations in cells as compared to CT VS cells.

The RTL-W1 cell line is an interesting tool for research on the n-3 PUFA elongation and desaturation capacity in fish as it has been observed in the

present study and by Ferain *et al.* (2016) that these cells have the ability to convert ALA into EPA if the culture medium is enriched in ALA substrate. However, considering the absence of peroxisomes in RTL-W1 cells (Malhão *et al.*, 2013) and potential enzymatic losses, the bioconversion cannot be carried out until the DHA production (Ferain *et al.*, 2016). In RTL-W1 cells enriched in ALA, the supplementation of SES and, to a lesser extent, EPI have negatively impacted the fatty acid bioconversion capacity of cells. In contrast to our results, SES and EPI were found to stimulate the n-3 PUFA biosynthesis pathway in a primary culture of Atlantic salmon hepatocytes (Trattner *et al.*, 2008b; Schiller Vestergren *et al.*, 2011). The lower concentration in n-3 LC-PUFA and the higher concentration in ALA in the cells used in the present study, as compared to the reported primary culture of hepatocytes, may partially explain the contrasting results obtained. Indeed, the *fads2* gene expression is under regulation of Pparα and Srebp1c (Nakamura and Nara, 2004) and n-3 LC-PUFA is presumed to down-regulate the *fads2* expression through inhibition of the Srebp1c activation by DHA (Matsuzaka *et al.*, 2002; Nakamura and Nara, 2004) and down-regulation of Pparα by EPA (Coccia *et al.*, 2014). The supplementation in SES and EPI, which are known to activate the PPARα system in rodents (Kushiro *et al.*, 2002), could counteract the down-regulation and thus increase the *fads2* expression. Unlike n-3 LC-PUFA in the Atlantic salmon hepatocytes, the high amount of ALA in the present study is suggested to up-regulate the *fads2* gene expression through Pparα in RTL-W1 cells (Coccia *et al.*, 2014). The addition of SES and EPI, which are known to inhibit the Srebp1c system (Ide *et al.*, 2001), could therefore inhibit the up-regulation and reduce the bioconversion of ALA. The present study is the first one to assess the impact of linseed lignans and enterolignans on the n-3 fatty acid metabolism in a fish model. In contrast with sesame seed lignans and enterolignans, no apparent effect of the linseed lignans SDG and SECO was observed on the fatty acid metabolism of RTL-W1 cells. In contrast, the END supplementation negatively affected the bioconversion of ALA in RTL-W1 cells.

Overall, the present study concluded on the negative impact of SES and EPI on the fatty acid bioconversion in RTL-W1 cells enriched in ALA. These results contrast with those previously obtained in *in vivo* experiments and *in vitro* experiments with primary cultures with sesame seed lignans addition (Trattner *et al.*, 2008a; 2008b; Schiller Vestergren *et al.*, 2011; Trattner *et al.*, 2011; Schiller Vestergren *et al.*, 2013). The positive effects of SES and

EPI observed in *in vitro* experiments were based on cells with a fatty acid composition rich in EPA and DHA (Trattner *et al.*, 2008b; Schiller Vestergren *et al.*, 2011). However, in a context of dietary fish oil replacement, the inclusion of bioactive compounds to the fish diet will be realised with fish fed plant-derived oil diets, and fish tissues will consequently be reduced in n-3 LC-PUFA. The present results suggest that sesame seed lignans and enterolignans could negatively affect the fish fatty acid bioconversion capacity in *in vivo* experiments with fish fed a ALA-rich diet. However, in an *in vivo* experiment with rainbow trout fed a 100 % linseed oil diet supplemented with SES and EPI, an increased DHA proportion was reported in the fillet and no effect on fatty acid composition of the liver was observed (Trattner *et al.*, 2008a). Moreover, despite no effect on DHA, a reduced ALA percentage was observed in rainbow trout fillet with the presence of SES in a 100 % linseed oil diet (Schiller Vestergren *et al.*, 2013), potentially reflecting an increase of ALA bioconversion.

Other lipid modulators have been tested to increase the fatty acid bioconversion in fish. In rainbow trout, the inclusion of the co-factors iron, zinc, magnesium, niacin, riboflavin, pyridoxine and biotin at 100, 200, 300 or 400 % of their recommended dietary inclusion to a diet formulated with linseed and canola oils have been shown to induce positive effects by stimulating the fatty acid bioconversion at the molecular and enzymatic levels (Lewis *et al.*, 2013). Still in rainbow trout, the dietary supplementation of CLA to a fish oil diet enabled to increase the n-3 PUFA content in liver, mainly in EPA and DHA, although the expression of *fads2* decreased (Kennedy *et al.*, 2007). Considering the fish oil replacement by plant-derived oils in fish feed, the unsaponifiable fraction contains minor components which could interfere with the fatty acid bioconversion. For instance, the unsaponifiable portion of linseed oil consists mainly of sterols and tocopherols, about 440 to 588 mg/kg of tocopherols are found in linseed oil (Gunstone, 2011). In rainbow trout, it has been observed that the minor compounds of sunflower and linseed oils, together with SES, have an influence on the expression of *ppar* genes (Schiller Vestergren *et al.*, 2013).

To go further ahead on the present experiment and beyond, the quantification of lignans in the culture medium and in cells should be realised in order to (1) compare the absorption rates by RTL-W1 cells of the different lignans and enterolignans, (2) compare the effects among

treatments on a same basis and (3) evaluate the lignan proportions degraded in the medium or metabolised by cells.

The cytotoxicity of sesame seed and linseed lignans and of END has been tested once in the present study. It has been observed that the incubation of RTL-W1 cells with 50 µM ALA and 50 µM SES or END dissolved in DMSO slightly reduced the cell viability by 10 % whereas no effect of EPI, SDG and SECO was observed. The comparison of the present results with the literature is difficult since the cytotoxicity of lignans has only been reported for cancer and tumour animal cells. The lignans are suggested to reduce the cell proliferation, to inhibit the cell cycle and to induce the apoptosis of cancer and tumour cells (Sung *et al.*, 1998; Qu *et al.*, 2005; Akl *et al.*, 2013). Further investigations should therefore be performed to conclude on the potential cytotoxicity of lignans and enterolignans on RTL-W1 cells and identify the precise mechanisms involved in the lignan influence on fish cell viability. Moreover, a similar experiment to the present one should be realised with a reduced concentration in lignan or enterolignan.

Numerous studies could be carried out on the research of bioactive compounds stimulating the fatty acid bioconversion capacity of fish. Firstly, a research on the bioavailability of dietary plant lignans and other potential modulators would be needed to evaluate to what extent and in which form the ingested compounds reach the liver. This would, among others, help performing more appropriate *in vitro* studies on the fatty acid metabolism. An *in vitro* incubation of dietary modulators with a fish faecal inoculum should also be performed to mimic the fermentation process occurring in the fish gut. The identification via high performance liquid chromatography of the metabolites produced in the fermentation digesta would indicate the potential bioprocessing into other metabolites occurring before absorption by enterocytes.

Once the relevant metabolites of the bioactive compounds are selected, the screening of potential modulators of fatty acid metabolism should be continued. For that purpose, the use of the RTL-W1 cell line may be of great interest to obtain comparable, fast and inexpensive results. Since the liver cell fatty acid composition seems to highly influence the impact of modulators on lipid metabolism, the RTL-W1 cell fatty acid profile should be similar to the one recovered in the liver of a fish fed a ALA-rich diet deprived in n-3 LC-PUFA. Moreover, the screening could also be achieved

with primary cultures of hepatocytes isolated from fish previously fed a plant-derived oil diet, in order to be closer to the actual n-3 PUFA profile of fish liver and, even more importantly, to its enzymatic content.

In a third step, the bioactive compounds inducing positive results with *in vitro* experiments should be tested in *in vivo* experiments. A feeding trial with rainbow trout should be performed with diets formulated on ALA-rich lipid sources and the selected bioactive compounds. Finally, the assessment of the *fads2*, *elovl5*, *srebp1* and *pparα* gene expressions should be assessed in order to find out if the transcription rate of the target genes is responsible for the effects observed in terms of fatty acid profile.

In conclusion, the present *in vitro* results contrasted with the positive effects of sesame seed lignans previously observed on the fatty acid bioconversion with *in vitro* experiments or with salmonid feeding trials. More extensive research is required in order to find out the underlying mechanisms involved in the lignan impact on liver cell fatty acid metabolism. Moreover, the search for new potential lipid modulators which could be added to the fish feed to increase the fish ALA bioconversion capacity should be performed.

At the end of this work, it can be concluded that the interest of consuming farmed fish fed with plant derived oils is jeopardized by the increased C18 PUFA and reduced n-3 LC-PUFA contents in fish induced by the dietary replacement of marine fish oil by plant-derived oils. Nevertheless, the consumption of farmed salmonids fed ALA-rich plant-derived oils is still beneficial considering that (1) a significant amount of dietary ALA is bioconverted into n-3 LC-PUFA for species such as salmonids, which possess a relatively high fatty acid bioconversion capacity and (2) the increased ALA content in fish allows responding at a higher extent to the human ALA requirement, which are of 1 % of the total energy requirements (CSS, 2009; ANSES, 2016). Another relevant impact arising from the plant-derived oils inclusion to fish feeds is the increased content in n-6 C18 PUFA, especially LA, in fish tissues (Thanuthong *et al.*, 2011a; 2011b; Francis *et al.*, 2014). However, this increased LA content is counterbalanced by the dietary ALA supply, which is quite higher than LA considering the use of ALA-rich plant-derived oils such as linseed, *Camelina sativa* or echium oils (NRC, 2011). The dietary n-6 PUFA/n-3 PUFA ratio, and consequently the fish n-6 PUFA/n-3 PUFA ratio, can therefore be adequately balanced with an appropriate dietary lipid source selection.

Interestingly, despite the current reduction of fish meal and fish oil to the fish feeds, the farmed fish, especially salmonids, may continue to be an excellent source of n-3 LC-PUFA for the human consumers, in comparison with wild fish species and other animal and plant sources (Shepherd *et al.*, 2016; Sprague *et al.*, 2016). Indeed, although the relative proportions (in percentage) of EPA and DHA are higher in wild salmonid products, in absolute terms (in g per 100 g of fillet) the farmed salmonids fed on plant-derived oils have been shown to deliver about the double dose of EPA and DHA than their wild counterparts (Henriques *et al.*, 2014; Sprague *et al.*, 2016). Therefore, the recommended amount of 250 mg of EPA and 250 mg of DHA per day or 3.5 g per week (ANSES, 2016) could be achieved by eating two portions of 150 g of farmed salmon whereas five portions would be required considering wild salmon (Henriques *et al.*, 2014). However, in the study of Henriques *et al.* (2014), the dietary fish oil replacement by plant-derived oils was not total and the findings do thus not represent the situation of fish fed a 100 % plant-derived oil feed. The use of a 100 % plant-derived oil diet, even exclusively based on linseed oil, would probably not allow obtaining such conclusions. As an example, the fillet fatty acid composition of experimental fish fed LO at 15°C, as reported in Chapter 3, contained about 200 mg of EPA and DHA (about 40 mg of EPA and 160 mg of DHA) by 100 g of fillet. This amount represented about 60 % of the EPA and DHA contents reported for fish fed FO (about 60 mg of EPA and about 280 mg DHA by 100 g of fillet). In that experiment, the final fish weight was about 50 g and not of a marketable size. However, if we extrapolate for a fish reached a marketable size, one portion of fillet of 150 g of fish fed LO would provide us roughly with the daily requested amount of DHA. Considering two portions a week, this would represent 27 % of the weekly requested amount of DHA. The complete replacement of fish oil by plant-derived oils would thus need to be associated with additional means, such as the inclusion of bioactive compounds to stimulate the fatty acid bioconversion capacity of fish. Moreover, the inclusion of n-3 LC-PUFA-rich lipid sources could be an interesting mean in order to sustain the n-3 LC-PUFA content of fish fillet to the recommended amount for human consumers (Tocher, 2015).

The replacement of fish oil by plant-derived oils represents a beneficial aspect in terms of farmed fish load in organic and inorganic contaminants. Indeed, fish oil is a marine-derived ingredient and can contain relatively high levels of persistent organic pollutants (POPs), *e.g.* PCBs, and inorganic

contaminants, such as heavy metals. These are therefore present in the feed and, as a result, accumulate in farmed fish (Turchini *et al.*, 2009; Hixson, 2014). The inclusion of plant-derived oils in fish feed reduces therefore the level in these pollutants in feeds, and subsequently in the final product (Médale *et al.*, 2013; Nøstbakken *et al.*, 2015). For instance, in Atlantic salmon, a 61 % lower level in PCBs was reported in the fillet of fish fed a diet in which 60 % of the total dietary lipid was provided by linseed oil and 40 % by fish oil, as compared to fish fed a 100 % fish oil diet (Friesen *et al.*, 2008). Nevertheless, it is worth noting that the inclusion of plant-derived oils may lead to the presence of undesirable contaminants from terrestrial crop productions, such as pesticide residues, into the fish feed (Turchini *et al.*, 2009; Gunstone, 2011; Shepherd *et al.*, 2016).

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# Chapter 7

## Conclusion

In a changing environment, the aquaculture production will face several challenges. These include the supply of high nutritional value fish to human consumers and the development of efficient production processes adapted to warmer waters. In this context, the *in vivo* and *in vitro* experiments carried out as part of the present research led to the following conclusions:

- The water temperature increase induced by the global warming will negatively affect the fatty acid bioconversion capacity of rainbow trout at the molecular and enzymatic levels.
- The high basal fatty acid bioconversion capacity of rainbow trout is not improved when fry are severely depleted in n-3 PUFA.
- The sesame seed and linseed lignans do not enhance the ALA bioconversion in RTL-W1 cells enriched in ALA.

To still produce fish with high n-3 LC-PUFA content, the dietary fish oil replacement by alternative n-3 PUFA-rich lipid sources appears to be a promising approach. This will however most probably be associated with other approaches. Therefore, future research should be dedicated to (1) the optimisation of fish n-3 LC-PUFA biosynthesis and retention through dietary inclusion of ALA-rich lipid sources and fish genetic selection, (2) the inclusion of bioactive compounds to the fish feed in order to maximise the fish fatty acid bioconversion capacity, and (3) the inclusion of alternative sources of n-3 LC-PUFA into fish feed at the end of the grow-out period in order to maintain the n-3 LC-PUFA content of fish tissues.

Beyond the present work, numerous studies have been focused on the increase of the fish fatty acid bioconversion capacity and, despite the high variation in the parameters, tools and approaches selected, the increase of fish bioconversion capacity is still not sufficient to obtain a n-3 LC-PUFA-rich fish when fed a plant-derived feed. Therefore, the search has still to be engaged in the development of aquaculture processes providing n-3 LC-PUFA-rich fish, taking the environmental, economic and social aspects into account.