Fakulta rybářství
a ochrany vod
Faculty of Fisheries
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of Waters

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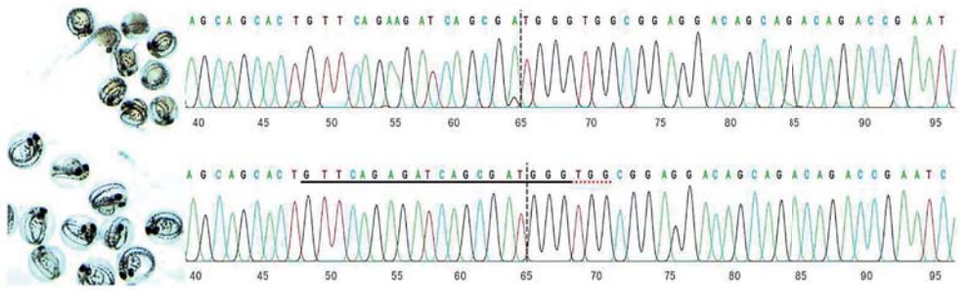
Regulation of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis in fish

Regulace biosyntézy vysoce nenasycených mastných kyselin (LC-PUFA) u ryb



Doctoral thesis by
Zuzana Bláhová

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"As for the future, it is not a question of foreseeing it, but of making it possible."

Antoine de Saint Exupéry, *Citadelle*, 1948

CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1. General introduction

The Intergovernmental Panel on Climate Change (IPCC) predicts in it´s Sixth Assessment Report on Climate Change 2022: Impacts, Adaptation and Vulnerability that in the 21st century, nutritional quality, when defined as the value of the product for the consumer´s physical health, growth, development and reproduction, is at a steady growing risk. Long-chain polyunsaturated fatty acid (LC-PUFA) biomolecules are important building blocks of fat and essential dietary components for humans and virtually all animals. According to some models announced, LC-PUFAs provisioning by ecosystems declines dramatically (Hixson and Arts, 2016; IPCC, 2022). Indeed, evaluations of natural ecosystems' capacity to produce LC-PUFAs are essential for pure and applied sciences.

1.1. Fatty acid biomolecules architecture

Fatty acids are aliphatic molecules composed of a hydrophobic tail (long non-polar hydrocarbon chain) attached to a hydrophilic head (polar carboxylic acid). When such molecules are placed in water, they aggregate spontaneously, arranging their hydrophobic portions to be as much in contact with one another as possible to hide them from the water while keeping their hydrophilic portions exposed. They tend to associate with other acyl chains or with hydrophobic regions of available proteins (Alberts et al., 2002; Lands, 2022).

Fatty acids rarely occur as free molecules in nature (non-esterified fatty acids, often called "free fatty acids") (Voet and Voet, 2004). Mostly, they occur in esterified forms as the major components of:

(i) fats (triacylglycerols, TAG), glycerol molecules, whose three hydroxyl groups are combined with the polar hydroxyl groups of fatty acid carbon chains (Fig. 1). These molecules readily bind to other fat molecules and form water-insoluble large spherical fat droplets in the cell cytoplasm;

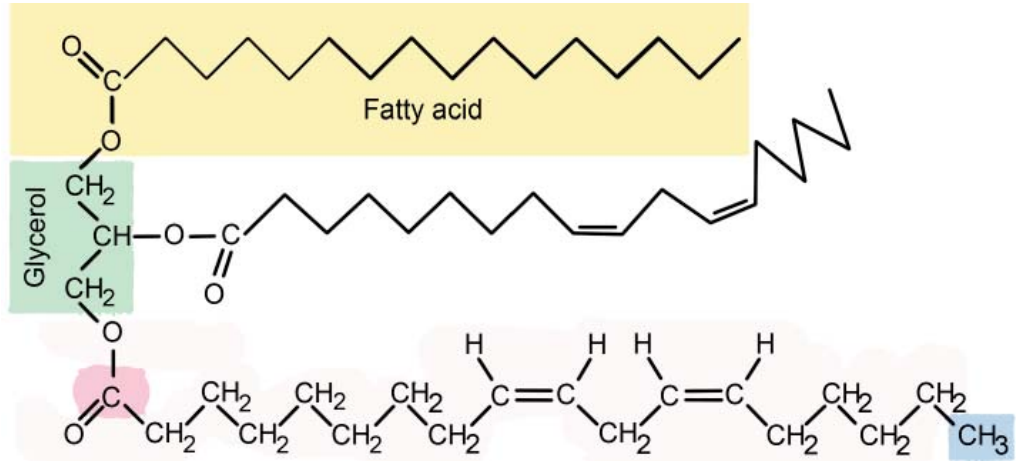


Figure 1. *The triacylglycerol (TAG) structure is composed of glycerol esterified to three fatty acids. This TAG has a 16C saturated fatty acid in the sn-1 position and two 18C polyunsaturated fatty acids in the sn-2 and sn-3 positions (https://veteriankey.com/fat-and-fatty-acids/).*

(ii) phospholipids, molecules of glycerol, which have a highly hydrophilic phosphate ester in the *sn*-3 position and in the *sn*-1 and *sn*-2, the glycerol backbone is linked to hydrophobic fatty acids. They form bilayer structures with outer hydrophilic ends in contact with water, and their inner hydrophobic parts, shielded from the water (Fig. 2). The property of self-sealing lipid bilayers is the key role which makes phospholipids fundamental components of membranes in living cells (Alberts et al., 2002). Membranes separate individual cells from their environments and compartmentalise the cell interior into structures that carry out special functions.

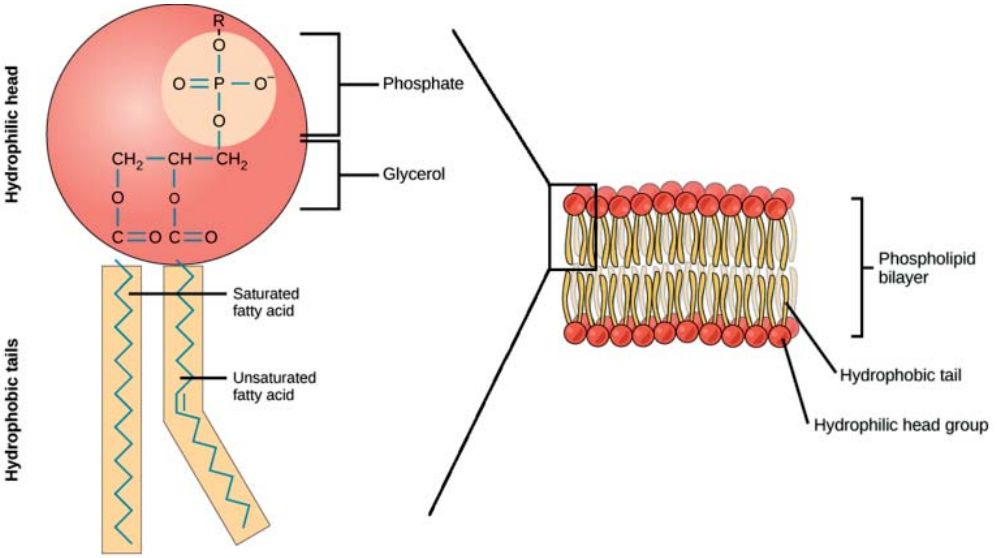


Figure 2. *Scheme of phospholipid molecule with its hydrophilic head and hydrophobic tails and as a constituent of a phospholipid bilayer (https://www.khanacademy.org/science/biology/macromolecules/lipids/a/lipids).*

(iii) sterol esters, which are created when the carboxy group of any fatty acid condensates with sterol. They are minor constituents of oils and fats.

1.2. Fatty acid nomenclature

Fatty acids (FAs) are carboxylic acids with the general formula R-COOH, with R representing $CH_3(CH_2)_n$ in the case of fully saturated fatty acids, and with R representing $CH_3(CH_2)_mCH{=}CHCH_2(CH_2)_n$ in the case of unsaturated fatty acids, which contain one or more double bonds between carbon atoms in the hydrocarbon chain. The *n* reaches from 0 to over 40. Aliphatic fatty acids are named by dropping the final ´e´ from the parent alkane and adding the term ´oic acid´ to the root (Voet and Voet, 2004; Brondz, 2005).

Designation for the systematic names of fatty acids is A:B ωC, where A is the number of carbon atoms, B the number of double bonds, and C the position of the double bond from the aliphatic end of the molecule (Bianchi and Bauer, 2011).

For unsaturated fatty acids, the positions of double bonds can be designated in two alternative ways. In the ω (or n-) nomenclature, the double bond position is counted from the methyl group. The older Δ nomenclature caunts the position of the double bond from the carboxyl group. Thus, for example, linoleic acid (LA) can be designated as 18:2n-6 or 18:2ω6

or 18:2$\Delta^{9, 12}$, a fatty acid with two double bonds with the first being at the carbon adjucent to the methyl end of the molecule or at carbons 9 and 12 when caunted from the carboxylic end of the fatty acid molecule in the Δ nomenclature (Fig. 3, Fig. 4).

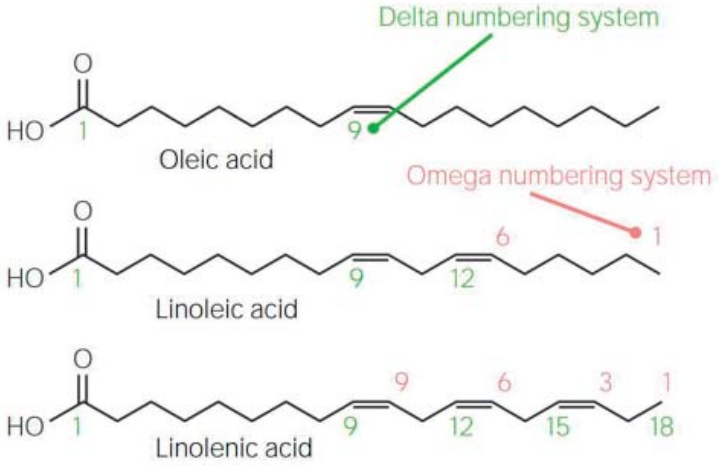


Figure 3. *Principle of fatty acid numbering (https://www.lecturio.com/concepts/fatty-acid-metabolism/).*

The fatty acids with a chain length of C16 and C18, saturated and unsaturated, are the most common fatty acids in nature (Cranwell et al., 1982; Bianchi and Bauer, 2011). Fatty acids with C18 polyunsaturated chains are indispensable biomolecules for virtually all animals. Fatty acids counting 20 and more carbons and more than two double bonds in their poly carbon chain are called long-chain polyunsaturated fatty acids (LC-PUFAs), from them eicosapentaeonic acid (EPA, C20:5ω3), docosahexaenoic acid (DHA, C22:6ω3) and arachidonic acid (AA, C20:4ω6) are especially physiologically important, hence, the most studied LC-PUFA biomolecules linked to human health (Zárate et al., 2017; Van Dael, 2021).

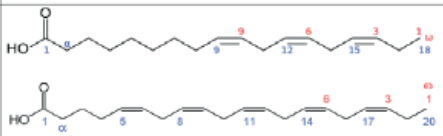
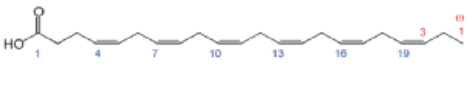
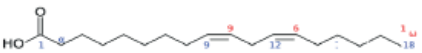
Common Name	IUPAC	Omega Numbering	Delta Numbering	Chemical Structure
α-linolenic acid (ALA)	9,12,15-octadecatrienoic acid	C18:3ω3	C18:3Δ9,12,15	
Eicosapentaenoic acid (EPA)	5,8,11,14,17-EPA	C20:5ω3	C20:5Δ5,8,11,14,17	
Docosahexaenoic acid (DHA)	4,7,10,13,16,19-DHA	C22:6ω3	C22:6Δ4,7,10,13,16,19	
Linoleic acid (LA)	9,12-octadecadienoic acid	C18:2ω6	C18:2Δ9,12	
Arachidonic acid (AA)	5,8,11,14 -Eicosatetraenoic acid	C20:4ω6	C20:4Δ5,8,11,14	

Figure 4. *Naming of fatty acid molecules.*

1.3. Fatty acid synthesis

De novo fatty acid synthesis is a critical anabolic pathway in most organisms. In nature, two fundamentaly different pathways evolved to build fatty acids, aerobic and anaerobic (Gemperlein et al., 2014; Jovanovic et al., 2021).

1.3.1. Aerobic fatty acid synthesis

In biochemistry, fatty acid synthesis is the creation of fatty acid from acetyl-CoA and NADPH through the concerted action of two enzymes: acetyl-CoA carboxylase and fatty acid synthase (FAS). Under aerobic conditions, the fundamental process is highly conserved among species and largely similar among plants and animals. It takes place in chloroplasts of photosynthetic cells and cytosol of animal cells (Akoh and Min, 2008).

Basically, FAS catalyses a repeating four-step reaction cascade by which the fatty acyl chain is extended by two carbons, at the carboxyl end, in every passage through the cycle, explained in detail by Voet and Voet (2004). The main product of these reactions is palmitic acid C16:0, the most common saturated fatty acid in lipids of animals and most plants. It is the first fatty acid produced during fatty acid synthesis in humans and the fatty acid from which longer fatty acids can be produced. Once C16 fatty acid has been formed, it can undergo many modifications, including further elongations and/or desaturations (Voet and Voet, 2004).

Desaturations (insertions of double bonds between two adjacent carbons) are being catalysed by oxygen-dependent desaturase enzymes (FADs), which ultimately consume NADH (Aguilar and Mendoza, 2006). To become desaturated, a fatty acid must be transferred from the cytosol into the endoplasmic reticulum within the cell (Li et al., 2016; LaBrant et al., 2018). In plant cell, the farther formation of C18 is conducted in two parallel pathways – the ´prokaryotic´ one in plastid (He et al., 2020) and ´eukaryotic´ one in endoplasmic reticulum. A scheme representative for fatty acid biosynthesis in a primary producer is given in Fig. 5. Typically, FADs are site-specific (Buček et al., 2020). That means that they recognize the order of its carbon in the fatty acyl carbon chain. For example, Δ9 FAD is specific for introducing a cis-double bond at the Δ9 position in the poly carbon chain (Fig. 3), in which a saturated fatty acid molecule is being modified into a monounsaturated fatty acid molecule (Pelley, 2012).

There are some differences in LC-PUFA biosynthesis between organism species. In primary producers (and as recently confirmed in some invertebrates (Kabeya et al., 2021; Tan and Zheng, 2021; Tan et al., 2022; Monroig et al., 2022)), desaturations are catalysed by Δ9 FADs, and further desaturations can be performed by Δ12 or Δ15 FADs. Thus, oleic acid can be converted to linoleic acid, the founder of the LC-PUFA biomolecules of ω6 series and then to α-linolenic acid, the founder of ω3 LC-PUFA. In contrast, no living vertebrate has been shown to have enzymes which could desaturate fatty acid chains beyond the 9–10 position of the poly carbon fatty acyl chain. That is because vertebrate genomes do not contain genes for Δ12 or Δ15 desaturases, indeed, they can not produce LC-PUFAs *de novo* (Lee et al., 2016).

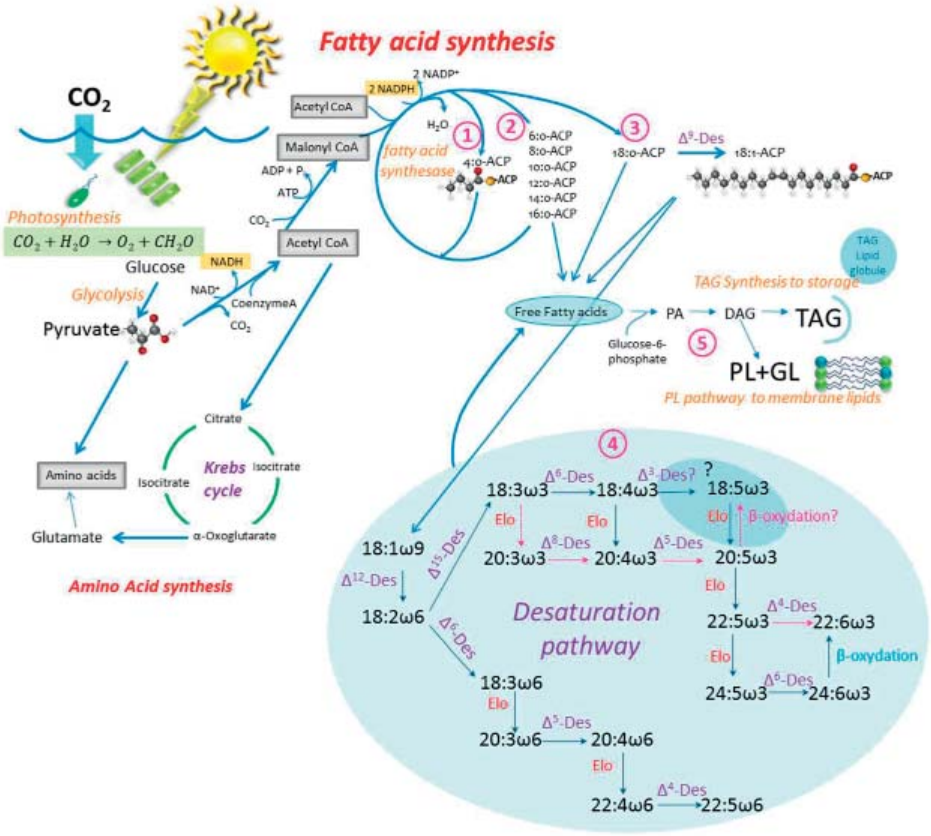


Figure 5. *Scheme of the de novo fatty acid synthesis example in the cell of a primary photosynthetic producer in aquatic ecosystems (Jónasdóttir, 2019).*

1.3.2. Anaerobic fatty acid synthesis

For long time, it has been assumed that all bacterial eicosapentaenoic acid (EPA, C20:5ω3) and docosahexaenoic acid (DHA, C22:6ω3) production arises aerobically by the action of the oxygen- dependent FAS enzymes. However, in some bacteria (Gemperlein et al., 2014, 2018) and unicellular eukaryotic microalgae (Alleman and Allen, 2018), it has been discovered relatively recently that the pathway of fatty acid synthesis is not integrated with FAS. Instead, it runs anaerobically by the use of a polyketide synthase (PKS)-like enzyme complex, to produce PUFAs *de novo* as explained in detail by Gemperlein et al. (2018). The advantage of this rout is, that it requires half less NAD(P)H than the aerobic route. Research of PKS-like PUFA synthesis attracts genetic ingeneers, who see the potential of PUFA-producing microbes to use them for industrial biotechnology with the aim to help fill the gap between PUFA biomolecules supply and demand in nutrition (Jovanovic et al., 2021). This thesis focuses on the aerobic route present in vertebrates including humans and fish.

Linoleic acid (LA, 18:2ω6) and α-linolenic acid (ALA, 18:3ω3) are essential nutrients for vertebrates, which are absolutely dependent on lower trophic levels to provide them. Once consumed, C18 precursors can undergo further modifications in a set of desaturation and elongation reactions into longer and more unsaturated LC-PUFAs. However, the endogenous

LC-PUFA biosynthesis is usually referred as to be limited (Cook and McMaster, 2004; Brenna et al., 2009; Parrish, 2009). This pathway is generally considered to serve as a compensatory apparatus helping to maintain homeostasis under fluctuating environmental conditions (Mock et al., 2019). There is of high importance for health maintenance, well-being and good reproduction performance to keep an adequate amount and balanced composition of LC-PUFAs in body tissues (Arts et al., 2001; Coste et al., 2010; Nehra et al., 2012). LC-PUFAs remain indispensable nutritional components and are widely accepted as semi-essential nutrients. Therefore, health authorities such as FAO and WHO have made recommendations for dietary intakes (Coste et al., 2010; WHO; 2008).

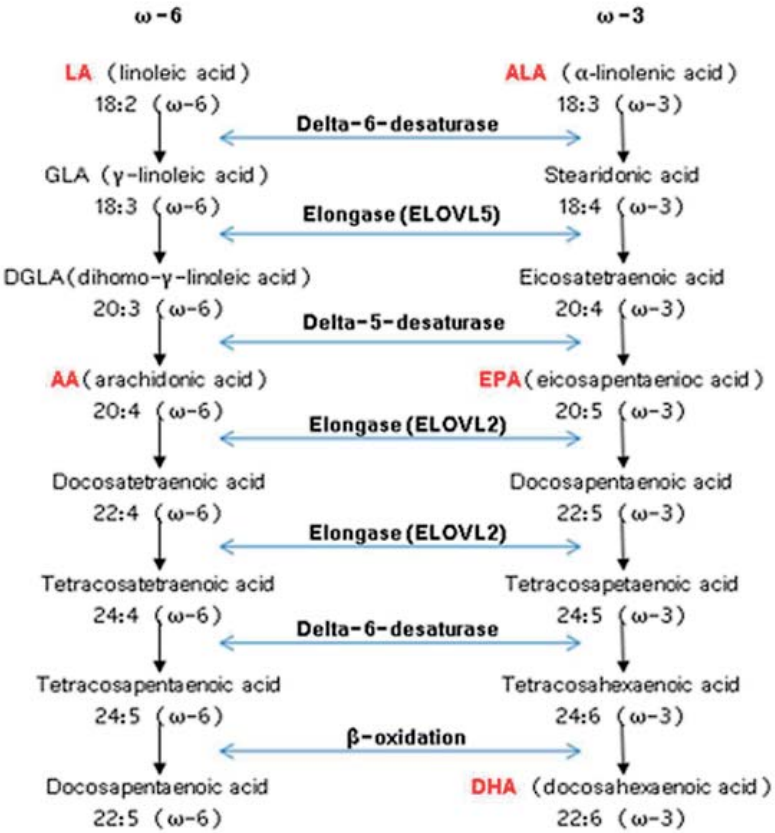


Figure 6. *The main endogenous pathway of ω6 and ω3 LC-PUFA biosynthesis from dietary essential PUFAs (adopted and changed from https://www.gbhealthwatch.com/Science-Omega3-Omega6.php).*

1.4. LC-PUFAs in food webs and warmer worlds

LC-PUFAs arise at the basis of grazing food webs in aquatic ecosystems (Bell and Tocher, 2009; Hixson and Arts, 2016; Li-Beisson et al., 2019; Zulu et al., 2018), where substantial amounts of LC-PUFAs are provided by single-cell microorganisms such as photosynthetic microalgae, heterotrophic protists and bacteria (Brett and Müller-Navarra, 1997), as well as macrophytes (Galloway et al., 2012). Recently, multiple invertebrates have been confirmed to produce PUFAs *de novo* and further biosynthesise them into LC-PUFAs similarly to single-

cell microorganisms (Kabeya et al., 2021; Tan and Zheng, 2021, Tan et al., 2022; Monroig et al., 2022). For a long time, aquatic ecosystems were considered the only primary source of LC-PUFAs. However, in terrestrial systems, belowground invertebrates can well be a source supporting aboveground biota (Menzel et al., 2018). Interlinks from terrestrial to aquatic ecosystems are biochemically totally different from *vice versa*. While biomass rich in LC-PUFAs is transferred to land, e.g., by trophic relationships, the link from terrestrial to aquatic ecosystems provides recalcitrant plant carbon, mainly devoid of essential nutrients (Ruess and Müller-Navarra, 2019).

The anthropogenic climate changes influence food webs via altered input and transfer of essential biomolecules, but separating the effects of nutrients, CO_2 and warming are not trivial (Tan et al., 2022). Little is known about the environmental distribution of essential FA and how ecosystem functions are constrained by their bioavailability. Essential biochemical resources can directly influence a consumer´s growth and reproduction and, in turn, trophic relationships, including food intake or metabolic requirements. Thus, their availability can act as a selection pressure on the evolution of consumer populations (Ruess and Müller-Navarra, 2019). There is mounting evidence of species redistribution as the climate warms (Lenoir et al., 2020). To improve resilience to warming metabolic adjustments can be crucial. Niche shifts via physiological adjustments in response to environmental change are a critical and rapid mechanism by individuals to reduce extinction risk (Oellermann et al., 2022), and energy metabolism plays an important role in this context. An experimental simulation of subsidies from a degraded aquatic food web caused, in comparison to a control diet, a change in the fatty acid composition and, importantly, a decrease of DHA content in the muscle and brain of juvenile salmon (*Salmo salar*) (Závorka et al., 2021).

1.5. Genes of LC-PUFAs biosynthesis as key players in environmental adaptations

Recently, it has been confirmed in fish that the *fads2* gene coding for FADS2 desaturase is the key metabolic gene important for overcoming the nutritional constraints associated with colonisations of nutritionally pure water environments. It has been directly demonstrated that the Pacific Ocean three-spined stickleback (*Gasterosteus aculeatus*), primarily marine or anadromous fish with a higher *fads2* copy number, had a higher physiological ability to survive on DHA free diets. That enabled the fish to colonise nutritionally pure freshwaters in LC-PUFAs when new freshwater habitats emerged after glacial retreat (Ishikawa et al., 2019). Even in the human population, *fads* showed strong signatures of selection in Greenland Eskimos colonising polar regions (Dyerberg et al., 1978; Ishikawa et al., 2019).

DHA pure diets are one of the biggest challenges of human nutrition. It has been suggested that humans evolved on a diet with a low ω3/ω6 ratio, close to 1:1, whereas in the modern Western diet, this ratio exceeds 1:15 (Simopolous, 2011). This dietary change has been suggested to lead to the complex of civilisation diseases (Aguilar and Mendoza, 2006; Zárate et al., 2017). The presence of the endogenous LC-PUFAs biosynthesis has been confirmed in humans and could be hypothesised to play a role as a compensational apparatus to help to maintain homeostasis under fluctuating nutritional conditions. However, many studies indicate that the endogenous conversion of essential ALA to DHA in humans is much lower and more limited than previously assumed (Burdge et al., 2002; Hussein et al., 2005), and as a consequence, sources of ω3 LC-PUFAs such as fish should be included in the human diet for general health (Plourde and Cunnane, 2007; Bradbury, 2011; Zárate et al., 2017).

Fish, being considered the main source of health-promoting molecules ω3 LC-PUFAs for humans on the global scale (Gladyshev et al., 2015; Tocher et al., 2019) are dependent on

LC-PUFAs provisioning from lower trophic levels. The current world capture fishery production cannot be increased, and aquaculture is expected to continuously grow to deliver food to humans. Here this can be seen as a paradox. While aquaculture has increasingly become the source of EPA and DHA for humans, it has also, at the same time, become the most significant consumer of the world´s available supply of EPA and DHA. Currently, there is a persisting question to be solved, how to bridge the gap between LC-PUFAs supply and demand.

Chapter 2 of this thesis brings additional and more detailed information on this hot topic and concentrates on the first enzymatic steps of essential C18 substrates into subsequent downstream products catalysed by fatty acid desaturases (FADS2). It reviews available relevant literature and assesses FADS2 enzyme structure-function properties in fish in the context of environmental adaptations and as a target for genetic engineering.

Successful mass production of aquaculture species requires the provision of sufficient numbers of high-quality gametes (Brooks et al., 1997; Bobe and Labbe, 2010), whereby LC-PUFAs delivered by the diet in adequate amounts and composition play significant roles. LC-PUFAs affect spawning performance and egg quality (Fernández-Palacios et al., 2011; Izquierdo et al., 2001; Migaud et al., 2013). A diet deficient in ω3 LC-PUFA had a lower fecundity and egg viability in european sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) broodstock (Fernández-Palacios et al., 1995; Valdebenito et al., 2015). Improved fecundity, fertilisation, hatching and larval survival rates were observed when high ω3 LC-PUFA levels were administered to yellowfin sea bream (*Acanthopagrus latus*) and flame angelfish (*Centropyge loriculus*) broodstock. However, excessive levels of ω3 LC-PUFAs in the broodstock diet negatively affected larval survival, as shown in gilthead seabream (Fernández-Palacios et al., 1995). Improved fertilisation rate, relative fecundity, and egg and larval quality parameters were observed in greater amberjack (*Seriola dumerili*) when broodstock was fed adequate level of ω3 LC-PUFAs (Sarih et al., 2020). In order to determine the optimum requirements of dietary ω3 LC-PUFAs for broodstock fish, it is crucial to understand how egg quality is affected by ω3 LC-PUFAs. In contrast to marine fish (Agaba et al., 2004; Tocher et al., 2003; Tocher, 2010), previous studies have confirmed that endogenous LC-PUFAs biosynthesis is functional in many freshwater fish (Tocher, 2010).

Chapter 3 of this thesis aims to investigate a direct functional link between *fads2* gene and reproduction success in zebrafish (*Danio rerio*) by the use of the modern genome editing tool clustered regularly interspaced short palindromic repeats-associated CRISPR/Cas9 technology.

1.6. Objectives of the thesis

The thesis investigates the endogenous biosynthesis of LC-PUFAs in fish. In the context of growing aquaculture demand and dwindling LC-PUFA supply due to anthropogenic factors there is of urgent need to define enzymatic steps regulation of which could provide advantage to some fishes to survive and reproduce under conditions in the changing warming world. The objectives of the thesis are:

1. to summarize the currently available literature on LC-PUFA biosynthesis to find out the key enzymatic steps playing roles fundamental to adaptation of fish to novel environmental conditions.
2. to perform functional characterization of an enzyme of LC-PUFA biosynthesis in a suitable fish model organism by the use of a reverse genetic approach and to study a phenotype related to reproduction.

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CHAPTER 2

ASSESSMENT OF FATTY ACID DESATURASE (Fads2) STRUCTURE-FUNCTION PROPERTIES IN FISH IN THE CONTEXT OF ENVIRONMENTAL ADAPTATIONS AND AS A TARGET FOR GENETIC ENGINEERING

Bláhová, Z., Harvey, N.H., Pšenička, M., Mráz, J., 2020. Assessment of fatty acid desaturase (Fads2) structure- function properties in fish in the context of environmental adaptations and as a target for genetic engineering. Biomolecules 10, 206.

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Review

Assessment of Fatty Acid Desaturase (Fads2) Structure-Function Properties in Fish in the Context of Environmental Adaptations and as a Target for Genetic Engineering

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Abstract: Fatty acid desaturase 2 (Fads2) is the key enzyme of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis. Endogenous production of these biomolecules in vertebrates, if present, is insufficient to meet demand. Hence, LC-PUFA are considered as conditionally essential. At present, however, LC-PUFA are globally limited nutrients due to anthropogenic factors. Research attention has therefore been paid to finding ways to maximize endogenous LC-PUFA production, especially in production species, whereby deeper knowledge on molecular mechanisms of enzymatic steps involved is being generated. This review first briefly informs about the milestones in the history of LC-PUFA essentiality exploration before it focuses on the main aim—to highlight the fascinating Fads2 potential to play roles fundamental to adaptation to novel environmental conditions. Investigations are summarized to elucidate on the evolutionary history of fish Fads2, providing an explanation for the remarkable plasticity of this enzyme in fish. Furthermore, structural implications of Fads2 substrate specificity are discussed and some relevant studies performed on organisms other than fish are mentioned in cases when such studies have to date not been conducted on fish models. The importance of Fads2 in the context of growing aquaculture demand and dwindling LC-PUFA supply is depicted and a few remedies in the form of genetic engineering to improve endogenous production of these biomolecules are outlined.

Keywords: fatty acyl desaturase; Δ6 - desaturase; long-chain polyunsaturated fatty acid; LC-PUFA; ω3; ω6; EPA; DHA; AA; essential fatty acid; health; fish; transgene

1. Introduction

Fatty acid desaturase 2 (Fads2) is an endoplasmic reticulum membrane bound protein which acts as the first enzyme in the biosynthesis of long chain (≥ C_{20}) polyunsaturated fatty acids (LC-PUFA). This pathway includes physiologically important eicosapentaenoic acid (EPA, ω3-20:5^{5,8,11,14,17}), docosahexaenoic acid (DHA, ω3-22:6^{4,7,10,13,16,19}), and arachidonic acid (AA, ω6-20:4^{5,8,11,14}) which are produced from the shorter and lower level polyunsaturated fatty acids (PUFA) α-linolenic acid (ALA, ω3-18:3^{6,9,12}) and linoleic acid (LA, ω6-18:2^{9,12}). Human and many fish genomes encode for Fads2 as well as for some other enzymes acting in the LC-PUFA biosynthetic pathway, namely Fads1, elongase 5 (Elovl5), elongase 4 (Elovl4) or elongase 2 (Elovl2). LC-PUFA are often referred as conditionally essential nutrients, meaning that however the organism could be capable to produce

them, this endogenous production is insufficient to meet demand, hence, LC-PUFA biomolecules must be obtained through the diet. Endogenous production is hypothesised to serve as a compensation apparatus, which helps the organisms to maintain homeostasis under fluctuating environmental conditions and LC-PUFA availability. This could be the reason why marine fish, unlike freshwater fish, do not have the capability to produce LC-PUFA at a significant level [1] as a consequence of living in nutritionally rich oceans. In contrast to the conditional-essentiality of LC-PUFA, precursors of LC-PUFA, LA and ALA, cannot be created de novo in nearly any living animal, since their genomes do not encode for enzymes capable to create them (such would be methyl-end desaturases with Δ12 and Δ15 activity converting oleic acid (18:1n-9) into LA (18:2n-6) and ALA (18:3n-3)) [2]. Hence, animals are usually dependent on plants for providing double bonds in the Δ12 and Δ15 positions of the two major precursors of the ω6 and ω3 fatty acids LA and ALA [3]. These two fatty acids, therefore, are called essential fatty acids. In the literature, however, most often, the conditional-essentiality of AA, EPA and DHA for vertebrates and humans is not considered and with LA and ALA, these biomolecules are altogether called essential fatty acids (EFA).

The exogenous supply of EFA for many animals, including some omnivorous terrestrial animals and humans, is from aquatic ecosystems. In aquatic ecosystems, substantial amounts of EPA and DHA are provided by primary producers. Historically, the primary production of these biomolecules has been associated exclusively to single-cell microorganisms such as photosynthetic microalgae, heterotrophic protists and bacteria. Recently, however, multiple invertebrates, many of them representing abundant groups in aquatic ecosystems, have been confirmed to be able to produce PUFA de novo and farther biosynthesize them into ω3 LC-PUFA similarly to single-cell microorganisms [4,5]. Once synthesized by microalgae or invertebrates, these biomolecules are transferred through trophic webs to organisms of higher trophic levels. Fish are considered as the best source of ω3 LC-PUFA for humans. However, anthropogenic factors such as pollution, eutrophication, climate change or biological invasions threaten the LC-PUFA production by primary producers at present. World capture fishery production cannot be increased and aquaculture is expected to be continuously growing to deliver food to humans. Here, this could be seen a paradox. While aquaculture has increasingly become the major source of EPA and DHA for humans, it has also, at the same time, become the greatest consumer of the world's available supply of EPA and DHA. The problem of bridging the gap between supply and demand of LC-PUFA was very recently excellently reviewed by Tocher et al. [1]. Since LC-PUFA have been identified as globally limited nutrients, the ability of an organism to compensate for dietary deficiencies of LC-PUFA by enhanced activity of its endogenous biosynthesis is of great importance for human and animal health as well as for the maintenance of fish as an EFA source for the human diet and aquaculture food.

Although it has been confirmed that alterations in activities of elongases Elovl4 and Elovl5 catalysing subsequent steps in LC-PUFA biosynthesis can alter EPA, DHA and AA production by promoting various disease states [6], Fads2 is still commonly considered as the rate-limiting and the most important enzyme of LC-PUFA biosynthesis. Moreover, in light of some recent significant publications, Fads2 appears as an enzyme with far-reaching implications for environmental sustainability.

2. Significance and Essentiality of LC-PUFA Biomolecules

LC-PUFA are important components of fat and in higher eukaryotes confer fluidity, flexibility and selective permeability to cellular membranes. They greatly influence many physiological processes. Participation of LC-PUFA in several major human pathologies (inflammatory-autoimmune diseases, cardiovascular diseases, cancer and neurodegenerative disorders) has been reviewed recently by Zárate et al. [7]. The pivotal role of lipids as an essential dietary component is now widely accepted; however, many decades of research have gone into this conclusion, which has recently been very well reviewed by Spector and Kim [8]. Fish played an essential role in coming to this conclusion. The essentiality of any fatty acid biomolecule was, for the very first time, reported in what was at the time highly controversial scientific work of George Oswald Burr in 1929 [9,10]. He and co-workers demonstrated that LA and ALA rescued the growth retardance phenotype in rats fed fat-free diet and found the first

clue that LA is a precursor of AA. The concept of essential fatty acids appeared [11]. But it was before chain desaturation or elongation of fatty acids had been demonstrated and the authors wondered how two double bonds + two double bonds could equal four [12], as was elucidated later in series of studies conducted by Mead et al. [13]. The linkage between LA, its ω6 fatty acid desaturation and elongation products, and the formation of prostaglandins as biomediators was reported by Bergström et al. [14,15], who stated" … *the symptoms of essential fatty acid deficiency at least partly are due to an inadequate biosynthesis of the various members of the prostaglandin hormone system.*" The pathway through which ALA is converted into EPA and DHA was determined in 1960 by Klenk et al. [16]. Noteworthily, no important functions were attributed to ω3 fatty acids for a long time. However, this changed in 1968 when Dr. Jørn Dyerberg made the remarkable discovery that fats in the diet of Greenland Eskimos comprised mainly of fish were associated with a lowered risk of cardiovascular diseases, concretely, that plasma of these people contained large amount of ω3 fatty acids and their phospholipids contained high levels of EPA, but very little AA [17]. Their conclusion was that EPA protects against cardiovascular diseases [18]. Without a doubt, the paradigm had been changed and fatty acids were no more considered to fulfil the only function in energy storage. Widespread interest was awoken in investigations of unsaturated fatty acids as biomolecules indispensable for health. Numerous global and national health agencies and associations and government bodies have produced many recommendations for EFA intake for a healthy human diet through fish consumption. With the advent of molecular and genetic technologies, there appeared much evidence that a balanced abundance of EFA is a prerequisite for health and disease prevention in humans [19–21]. Meeting the dietary demands of a burgeoning human population with a correct dietary balance of EFA and at levels required for normal health and development has become a major challenge. It has been clear that understanding the molecular basis of LC-PUFA biosynthesis would underpin efforts to meet this challenge. Various strategies of human populations regarding EFA metabolism have been shown by Gladyshev and Sushchik this year [22]. Studies performed in fish are advantageous mainly because there is wide variation between fish species in their ability to biosynthesize LC-PUFA, probably as a consequence of inhabiting widely different environments. Comparisons of their genomes and expression levels of genes encoding key elements in the LC-PUFA biosynthetic pathway between species have been promising to increase knowledge of the molecular components of the pathway and of the molecular genetic basis of phenotypic variation in LC-PUFA biosynthesis.

3. Fads2 in LC-PUFA Biosynthesis

The biosynthesis of $C_{20\text{-}22}$ LC-PUFA involves alternating steps of desaturation (introduction of an additional double bond) and elongation (addition of two carbons) of the dietary essential C_{18} fatty acids LA and ALA [1]. Firstly, in the biosynthesis of LC-PUFA, Δ6 Fads2 desaturase converts dietary obtained LA (18:2n-6) and ALA (18:3n-3) into gamma-linoleic acid (GLA) and stearidonic acid, respectively. Subsequently, in the biosynthesis of LC-PUFAs of ω3 series, it converts tetracosapentaenoic acid into tetracosahexaenoic acid which is then converted to DHA. Enzymatic steps in the biosynthesis of LC-PUFAs in vertebrates are shown in Figure 1 [23–30]. AA and EPA are biosynthesized in the same pathway in which LA and ALA substrates compete of the same enzymes, respectively. The pathway revealed from studies in vertebrates are the so-called "Δ6 pathway" (Δ6 desaturation–elongation–Δ5 desaturation) and the "Δ8 pathway" (elongation–Δ8 desaturation–Δ5 desaturation). DHA is achieved downstream in the biosynthesis of LC-PUFA from EPA via two alternative routes. Either, two consecutive elongations of EPA produce tetracosapentaenoic acid (TPA, 24:5n-3), which then undergoes a Δ6 desaturation to tetracosahexaenoic acid (THA, 24:6n-3), the latter being β-oxidised to DHA in peroxisome organelles following the translocation from endoplasmic reticulum, the so-called "Sprecher pathway" identified in mammals [24,31], or, the direct Δ4 desaturation of docosapentaenoic acid (DPA, 22:5n-3) into DHA via the 'Δ4 route´. The first Fads2 gene with Δ4 activity was identified in the marine herbivorous fish *Siganus canaliculatus* [32]. It was not only the first enzyme with this activity among fish, but it was the first discovered case in all vertebrates. The discovery indicated that there

exists another possible mechanism for DHA biosynthesis, a direct route involving elongation of EPA to 22:5n-3 followed by Δ4 desaturation. If both DHA routes were coexist, this would represent a clear advantage for satisfying DHA requirements through endogenous production. After further identification of 11 teleost species having a putative Δ4 Fads2 by Oboh et al. [33], it was made clear that the direct Δ4 pathway is more widespread among teleost fish than initially believed.

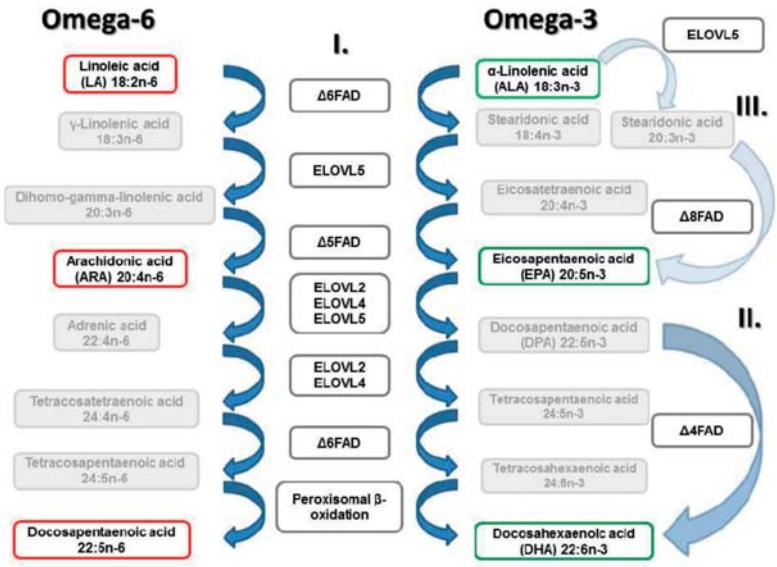

Figure 1. Biosynthetic pathways of long-chain (≥ C_{20}) polyunsaturated fatty acids (LC-PUFAs) of ω3 and ω6 families from dietary essential α-linolenic (ALA, 18:3n-3) and linoleic (LA, 18:2n-6) acids, respectively, by elongation and desaturation reactions. Adapted from (Carmona-Antoñanzas et al., 2011; Monroig et al., 2011; Sprecher, 2000; Voss et al., 1991); modified after (Trattner, 2009; Vestergeren, 2014; Yan, 2016).

4. Fads Gene Repertoire in Fish

There is a fundamental difference between fish and mammals regarding the gene repertoire encoding for enzymes performing desaturation activities needed for LC-PUFA biomolecules production. In contrast to mammals, where distinct separate genes *Fads1* and *Fads2* encode enzymes Fads1 and Fads2 with appropriate specificities Δ5 and Δ6 [34], respectively, in fish, *Fads1* gene has been lost during the evolution. As a result, all desaturation steps of the LC-PUFA biosynthetic pathway in fish are catalysed by Fads2 enzymes, exhibiting different Δ activities which can be overlapping to some extent.

Until recently, this scenario was generally accepted with no exceptions. Accordingly, one single Δ6 Fads2 appears most often [35–38]. Less often, a separate Δ6 Fads2 and Δ5 Fads2 paralogues appeared, such as in Atlantic salmon (*Salmo salar)* [39,40], or more than one single Δ6 Fads2 paralog are present such as in common carp (*Cyprinus carpio*) [41] and recently confirmed in numerous Osteoglossomorpha species [42]. In some teleosts studied, Δ6 Fads2 had measurable levels of Δ5 activity [37] or Δ8 activity [42,43]. A single bifunctional Δ6/Δ5 Fads2 acts in zebrafish (*Danio rerio*) [44] which was the first functionally characterized fish desaturase and for some time, it has been considered as an exception, not only in fish but in vertebrates in general. Later, two desaturases from marine rabbitfish (*Siganus canaliculatus*) were functionally characterized, one of which was shown to be Δ6/Δ5 bifunctional and the other Δ5/Δ4 bifunctional [32]. There exist extreme exceptions as well, represented by teleosts lacking *Fads*-like genes in their genomes, namely pufferfish *Takifugu rubripes* and *Tetraodon nigroviridis* [42].

The Atlantic salmon Δ6 and Δ5 *Fads2* cDNAs are very similar, sharing greater than 95% nucleic acid identity, indicating the presence of a recently duplicated locus, probably as the result of the recent salmonid whole genome duplication event [40].

The property of fish Fads2 exhibiting a more varied spectrum of Δ activities towards substrates has been hypothesized by Castro et al. [45] as a result of a functionalization process that occurred in response to dietary availability in natural pray. Functionally characterized Fads2 in numerous teleosts and all their activities determined by heterologous expression in yeast are listed in recent review of Kabeya et al. [46]. However, the persisting lack of information in some teleost lineages, such as Elopomorpha, and other nonteleost lineages, such as Lepisosteiformes, Polypteriformes or Cyclosomata, has hampered the full comprehension of Fads enzymes function in fish for a long time. Current novel insights into the fish LC-PUFA biosynthesis have provided a study on Fads desaturases published by Lopes-Marques et al. [42]. Accordingly, two types of desaturase repertoire are confirmed to appear in teleost fish, separating Elopomorpha from the other living teleost lineages. The orthologous gene to *Fads1* has been found in Japanese eel (*Anguilla* japonica), an Elopomorpha teleost specie, and confirmed by heterologous expression approach in yeast that desaturates the corresponding fatty acid substrates in the Δ5 position as well as sharing the common structural features to mammalian Fads1 enzymes. Farther Fads1 have been identified in some representatives of ancient fish lineages such as the Senegal bichir (*Polypterus senegalus*) and spotted gar (*Lepisosteus oculatus*) by these authors [42].

Based on sequence and phylogenetic data, *Fads2* and *Fads1* genes have been deduced to originate from the vertebrate ancestor and *Fads1* seems to be lost in Teleostei lineages except in Elopomorpha. It could be hypothesized that some teleosts have generated a mechanism to overcome the bottleneck caused by the loss of Δ5 Fads1, since otherwise, they would not be able to convert PUFA to LC-PUFA. Such a mechanism would be *Fads2* gene duplication followed by the process of functionalization as most probably was the case in salmonids, whereby acquisition of Δ5 *Fads2* occurred in one of the several *Fads2* gene copies. Another example would be the zebrafish (*Danio rerio*) in which Δ6 Fads2 acquired the ability to desaturate even in the Δ5 position [42,44]. The loss of canonical *Fads1* gene followed by *Fads2* subfunctionalization that teleosts have undergone during evolution could be linked to and explain the higher plasticity with which fish produce LC-PUFA biomolecules in comparison to other vertebrates.

5. Fads2 Structure and Structural Implications of Substrate Specificity

Fads2 are modular proteins which characteristically have a cytochrome *b5*-like domain on the N-terminus and the main desaturation domain with three histidine-rich regions on the C-terminus [47–49]. The fusion of the cytochrome *b5*-like domain to the main desaturase protein domain enables the NADH cytochrome *b5* reductase to directly transfer electrons to the catalytic site of Fads2 via the cytochrome *b5*-like domain without the requirement for an independent cytochrome *b5* [50,51]. However, solid evidence has been provided that both the cytochrome *b5*-like domain of Fads2 and microsomal cytochrome *b5* are necessary in the process of Δ6 desaturation and that the microsomal cytochrome *b5* does not compensate for the role of cytochrome *b5*-like domain of Fads2, which is accompanied by highly conserved heme-binding HPGG motif of the cytochrome *b5*-like domain. Moreover, protein–protein interactions between Fads2 and microsomal cytochrome *b5* are required for proper Fads2 function [52]. Phylogenetic studies have shown that cytochrome *b5* domain from Δ6 Fads2 proteins form a single cluster which points to a single ancient fusion event that took place in the common ancestor of all eukaryotes [53].

As a hydrophobic membrane-bound protein, Fads2 is extremely recalcitrant to characterization by conventional biochemical methods. A three-dimensional structure of Fads2 by X-ray crystallography is missing to date. The only animal desaturase whose structure is known is the stearoyl-CoA desaturase with Δ9 desaturation activity for which crystal structures have been published in humans [54] and rats [55]. There are some characteristic features common to all desaturases. The amino acid sequences within the substrate binding channel in all membrane desaturases contain the three His-boxes which

histidine residues hold two irons in the active site. These histidine residues are of high evolutional conservation [56,57] and take place in very close proximity to the fatty acid substrate, referred to as "contact residues" [51]. Hydropathy analyses have shown that desaturases contain up to three long hydrophobic domains which are long enough to span the membrane bilayer twice whereas the His-boxes have a consistent positioning with respect to these potential membrane spanning domains. Sayanova et al. [56] undertook a massive motif analysis in more than fifty eukaryotic genomes, obtaining 275 desaturases, and reported the sequence logo representations of conserved histidine regions shown in Figure 2.

Figure 2. Sequence logo of histidine boxes in membrane associated front-end desaturases such as Δ6 Fads2. The high of letters corresponds to the occurrence probability. Adapted from (Sayanova, 2001). Modified after (Hashimoto, 2007).

The first report on the structural basis of the substrate specificity of a mammalian front-end fatty acid desaturase was published by Watanabe et al. in 2016 [58]. Using the crystal structure modelling of the human soluble stearoyl-CoA (Δ9) desaturase [54,55], these authors performed homology modelling and revealed that Arg216, Trp244, Gln245, and Leu323 are located near the substrate-binding site. They applied site-directed mutagenesis to create mutations in rat Δ6 Fads2 at those sites they had predicted to influence the enzymatic function. Then, they exchanged these amino acids accordingly to be the same as in the unique bifunctional Δ6/Δ5 Fads2 from zebrafish. They determined amino acid residues responsible for both switching and adding the substrate specificity of rat Δ6 Fads2. Additionally, they predicted tertiary structure of rat Δ6 Fads2. There is a very important outcome from their publication—that one single amino acid in the Δ6 Fads2 desaturase enzyme, when changed, has the potential to switch the specificity towards substrates.

This corresponded to results from investigations on sex pheromones in moths [59], where similarly, a change as small as a single amino acid substitution in a fatty acid desaturase enzyme was sufficient to change the enzymatic function of the whole enzyme, moreover, resulting in huge consequences in reproduction. According to their data delivered, MsexD2 desaturase gene in *Manduca sexta* duplicated during the evolution whereby one copy acquired one amino acid change. Then, in the process of neofunctionalization, this novel gene acquired the ability to introduce another double bond and produce an uncommon sex pheromone with significant implication in species reproduction.

Corresponding data were obtained by the study of Δ6 Fads2 from marine algae *Thalassiosira pseudonana* [60]. Mutation sites in Δ6 Fads2 from *T. pseudonana* were determined which appeared to induce a propensity for the enzyme to favor binding of a particular fatty acid, suggesting that these may be associated with substrate specificity. The focused primarily on desaturation kinetics and assessed molecular mechanisms underlying the catalytic activity of Fads2 in *T. pseudonana*, because this model organism offers the advantage of exhibiting a very high desaturase catalytic activity suitable for such studies. They divided the amino acid sequence of Fads2 into sections and at the same time, they have used Fads2 from *Glossomastix chrysoplasta*, which in the opposite has very low enzymatic activity, and divided it in the same sections. To determine the catalytic activity of each region, the corresponding regions of both Fads2 enzymes were systematically exchanged to construct recombinant swap genes, which were expressed in yeast. Kinetics of enzymatic catalytic activity of recombinant desaturase were measured as well as amino acid residues important for catalytic activity by the use of site-directed mutagenesis were determined. As a result, topology prediction was created depictured in Figure 3. Amino acid substitutions significantly impacted the desaturation catalytic efficiency providing a solid basis for in-depth understanding of catalytic efficiency of Δ6 Fads2 enzyme.

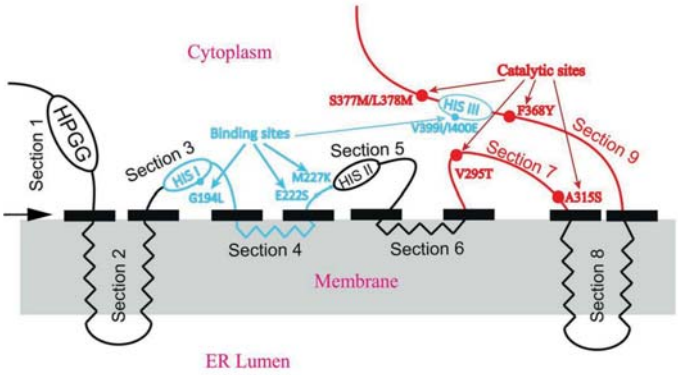

Figure 3. The predicted topology model of Δ6 Fads2. The black solid rectangles indicate boundaries of domains. Four alpha-helices span the membrane. The blue lines and blue dots indicate the areas and sites implicated in substrate specificity, red lines and red dots indicate the areas and sites important for catalytic activity, respectively. ER lumen: endoplasmic reticulum lumen. HIS I, HIS II and HIS III: histidine rich motifs. Modified from (Shi et al., 2018).

The abovementioned studies clearly demonstrated that the strictness of structure-function relationship of Fads2 enzymes might be enormous and acquired changes as small as one single amino acid of enzyme primary structure might have significant consequences for the organism studied which could be a general feature extrapolatable even to more diverse taxa such as fish. Comparative studies of highly effective and minimally effective LC-PUFA biosynthetic machineries either between more or less related species or occurring in one single species (as typically studied in salmonids [29,40,61] have justified, that this is a promising strategy with great potential to gain insight into the challenging LC-PUFA biomolecules research in fish.

The question why some species can survive in EPA and DHA poor environment and other even closely related species not, has been an attractive research topic in the very recent past. An interesting structure-function study performed by Xie et al. [62] has addressed that question by studying the *Fads2* promoter sequence. The binding site for stimulatory protein Sp1 has been found as lacking in the promoter of *Fads2* gene in marine teleost *Epinephelus coioides*. The authors speculated therefore, that the Sp1-binding site absence might be the main cause of the very low Fads2 expression in marine carnivorous teleost species. To test this hypothesis, they inserted the Sp1-binding site from the *Fads2* promoter sequence of the herbivorous *Siganus canaliculatus*, the first marine teleost demonstrated to have LC-PUFA biosynthetic ability, into the corresponding region of *E. coioides Fads2* promoter sequence. As expected, Δ6 desaturation activity increased significantly. The results provided direct evidence for the importance of the Sp1-binding site in determining *Fads2* promoter activity and indicated that its lack may be a reason for very low expression of Fads2 and poor LC-PUFA biosynthetic ability in *E. coioides*. The Sp1-binding site has been found as lacking in marine carnivorous fish *Gadus morhua* [36] as well as in *Dicentrarchus labrax* [63], while in *Oncorhynchus mykiss* its promoter activity was weaker [64].

6. Fads2 Copy Number Variation

The best was yet to come regarding studying marine vs. freshwater fish dealing with LC-PUFA poor food sources. Just a few months ago, Science released an exciting paper from Ishikawa et al. [65]. In this comprehensive study, the authors compared three-spined stickleback (*Gasterosteus aculeatus* species complex) which successfully colonized newly emerged freshwater bodies after glacial retreat with closely related marine Japan Sea stickleback (*G. nipponicus*) which had failed to colonize freshwater. They linked the colonization success to *Fads2* gene copy number, being higher in Pacific Ocean stickleback from the *G. acuelatus* complex. When transgenic Japan Sea stickleback overexpressing

Fads2 was made and fed only DHA-free *Artemia*, the Fads2 transgenics showed a higher survival rate and higher DHA content at 40 days after fertilization than the control GFP-transgenics. Moreover, *Fads2* gene linkage to X chromosome was confirmed, resulting in higher copy number in females. That fact is consistent with higher female survival observed. These results suggested that lower *Fads2* copy number may be a constraint to colonization of DHA-deficient freshwater niches by Japan Sea stickleback. These authors went deeper into the copy number assessing in the context of phylogeny and deduced a general mechanism: Higher *Fads2* copy number contributes to survival with DHA-free diets. Hence, *Fads2* was the metabolic gene important for overcoming the nutritional constraints associated with freshwater colonization in fishes. The authors mentioned the intriguing feature of the *Fads2* gene to make strong signatures of selection even in human such as in Greenland Eskimos which might be farther extrapolated to even more diverse taxa.

7. Fads2 Transgenes

The limited availability of LC-PUFA derived from fish represents the critical bottleneck in food production systems, one that numerous research institutions and aquafeed companies in this field are trying to overcome. Attempts to replace fish-derived LC-PUFA by plant derived alternatives often resulted in low quality products lacking the original content of these health promoting biomolecules [66]. This problem could be minimized by either feeding fish genetically modified plants for enhanced EPA and DHA production or by gene editing fish to be capable to produce endogenous LC-PUFA more effectively. Genetic engineering has been long been utilized as a strategy to increase natural productivity. Genetically engineered organisms could have the potential to reduce pressure on current LC-PUFA natural resources. The efforts and progress to develop transgenic plants as terrestrial sources of ω3 fish oils as well as advances in the field have been reviewed recently by Napier [67]. Transgenic fish have many potential applications in aquaculture, but the research also raised concerns regarding the possible risks to the environment associated with release and escape. A tabulated balance sheet of likely benefits and risks have been published by Maclean and Laight [68]. In this review, we focused on attempts to produce genetically modified fish with an enhanced content of ω3 LC-PUFA.

The first step to modifying the LC-PUFA biomolecules production pathway using genetic engineering was done in zebrafish [69], into which a gene for Δ5 Fads2 from masu salmon was introduced. The result demonstrated that masu salmon (*Oncorhynchus masou)* Δ5 Fads2 is functional in zebrafish and modifies its LC-PUFA metabolic pathway; hence, the technique could be applied to farmed fish to generate a nutritionally richer product for human consumption. The closely relative to zebrafish, the common carp (*Cyprinus carpio*) accounts for about 40% of the total global aquaculture production and could therefore deliver a significant amount of LC-PUFA if they were produced in their body. However, the content of EPA and DHA (mg g^{-1}) in muscle tissue of common carp is relatively low when compared to many other fish species, as revealed by recent meta-analysis data [70].

Some pioneering transgenesis experiments were carried out which reported trends towards increased ω3 LC-PUFA content in muscle of transgenic progeny—Δ5 *Fads2* from masou salmon driven by a β-actin promoter was introduced into common carp [71] and channel catfish (*Ictalurus punctatus*) [72] with the aim to improve ω3 LC-PUFA production. The results have shown promise for future work in this area, when utilizing homozygous transgenic individuals in contrast to the heterozygous individuals utilized in these studies. The effects of the transgene varied between common carp and channel catfish, being higher in common carp [72].

However, only a few month ago, *´Haiyouli´* construction was published [73], which, in Chinese, means "advantageous carp-like marine fish". This common carp was genetically modified with the aim to elevate production of ω3 LC-PUFA. The transgene used was a fish-codon optimized fatty acid desaturase (*fat1*) coding sequence originally from *Caenorhabditis elegans* driven by the 5´upstream regulatory region of common carp β-actin. Unexpectedly for the authors, under transgene expression fat accumulation of the internal organs decreased and in the liver tissues, *fat1*-transgenic common carp showed less accumulation of lipid droplets when compared with wildtype. However, the quantitative

RT-PCR results showed a 10.5-fold increase in Fads2 expression, a 6.5-fold increase in elongase 5 expression and a 3-fold increase in elongase 2 expression in the transgenic tissue, indicating stimulation of LC-PUFA biosynthesis by the expression of exogenous *fat1* desaturase. Interestingly, the transcription of acyl-CoA oxidase 3 increased by 8.2 in transgenic tissue, which perfectly explains the lipid content decrease in internal organs of genetically modified common carp. Intriguingly, the authors stated that the ω6 to ω3 ratio of their transgenic common carp (0.4) was even lower than that of the Atlantic salmon (0.58) reported by Henderson and Tocher [74]. For this reason, *Haiyouli* has been presented as a potentially ideal fish produced in modern society to balance the high ω6 to ω3 ratio of human diets. However, such a conclusion is questionable since they stimulate LC-PUFA biosynthesis by the expression of exogenous *fat1* at the expense of overall lipid biosynthesis. When total mass unit of LC-PUFA per mass unit of filet is calculated and compared to salmon, there may, in fact, be no relative advantage to consuming *Haiyouli*. Hence, it is rather a step forward on the way to constructing the ideal fish.

Successful production of DHA using *Fads2* transgenes has been reported in mammals. In Chinese hamster ovary cells, LC-PUFA-elevated production was achieved by heterologous expression of fish Δ4 Fads2 from *Siganus canaliculatus* with concomitant overexpression of Δ6 Fads2 and Δ5 Fads1 from mice. The authors stated that this new technology has been confirmed as very effective in high-level production of DHA from dietary ALA and provided a potential for the creation of new land animal breeds who could produce DHA abundantly in their related products [75].

If such solutions come into practice, this will have a positive effect in sufficient delivery of health promoting LC-PUFA to humans while at the same time, preserving wild fish populations.

8. Conclusions

Fads2 is a fascinating enzyme with far-reaching implications for both human health and environmental sustainability. It is clear that Fads2 has played an important role in the adaptations to novel environments throughout evolutionary history as differences in both gene expression and copy number have been reported across freshwater and seawater dwelling species. We have demonstrated the importance of this enzyme in the context of growing aquaculture demand and dwindling LC-PUFA supply and outlined a few remedies in the form of genetic engineering to improve endogenous PUFA production. By improving our understanding of Fads2, we can address major environmental concerns and break out of the cycle of exploitation that currently strains our wild fish reserves to feed the growing aquaculture sector.

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CHAPTER 3

PARTIAL *fads2* GENE KNOCKOUT DIVERTS LC-PUFA BIOSYNTHESIS VIA AN ALTERNATIVE Δ8 PATHWAY WITH IMPACT ON REPRODUCTION OF FEMALE ZEBRAFISH (*Danio rerio*)

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My share on this work was about 70%.

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Partial *fads2* Gene Knockout Diverts LC-PUFA Biosynthesis via an Alternative Δ8 Pathway with an Impact on the Reproduction of Female Zebrafish (*Danio rerio*)

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Abstract: The zebrafish (*Danio rerio*) genome contains a single gene *fads2* encoding a desaturase (FADS2) with both Δ6 and Δ5 activities, the key player in the endogenous biosynthesis of long-chain polyunsaturated fatty acids (LC-PUFAs), which serve essential functions as membrane components, sources of energy and signaling molecules. LC-PUFAs include the precursors of eicosanoids and are thus predicted to be indispensable molecules for reproductive health in virtually all vertebrates. In mice, an amniotic vertebrate, *fads2* deletion mutants, both males and females, have been confirmed to be sterile. In anamniotic vertebrates, such as fish, there is still no information available on the reproductive (in)ability of *fads2* mutants, although zebrafish have become an increasingly important model of lipid metabolism, including some aspects of the generation of germ cells and early embryonic development. In the present study, we apply the CRISPR/Cas9 genome editing system to induce mutations in the zebrafish genome and create crispants displaying a degree of *fads2* gene editing within the range of 50–80%. Focusing on adult G_0 crispant females, we investigated the LC-PUFA profiles of eggs. Our data suggest an impaired pathway of the LC-PUFA biosynthesis of the ω6 and ω3 series in the first-rate limiting steps of the conversion of linoleic acid (LA) into γ-linolenic acid (GLA), and α-linolenic acid (ALA) into stearidonic acid (SDA), respectively, finally resulting in bad-quality eggs. Our data suggest the existence of an alternative Δ8 pathway, which bypasses the first endogenous LC-PUFA biosynthetic step in zebrafish in vivo, and suggest that the zebrafish bifunctional FADS2 enzyme is actually a trifunctional Δ6/Δ5/Δ8 desaturase.

Keywords: *fads2*; Δ6/Δ5/Δ8 desaturase; long-chain polyunsaturated (LC-PUFAs) biosynthesis; CRISPR/Cas9; zebrafish (*Danio rerio*) reproduction; dihomo γ-linolenic acid (DGLA)

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1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFAs) are of high significance in healthy state maintenance and the reproduction success of virtually all vertebrates. Being important building blocks of lipids and playing vital roles as integral components of cell membranes, LC-PUFAs affect membrane fluidity, regulate ion channels, modulate endocytosis and exocytosis as well as hormonal activity, have immunological effects and influence gene expression during development. As precursors of eicosanoids, some LC-PUFAs are necessary for spermatogenesis and folliculogenesis [1–4]. In oocytes, the lipid metabolism is critical for their growth, fertilization, and the development of early embryos. The improper delivery of lipids to oocytes leads to abnormal oocytes, a low egg production rate, and low viability of embryos [5–7].

Vertebrates are usually capable of biosynthesizing LC-PUFAs to extend endogenously from essential precursors, in addition to the main acquisition via diet [8,9]. In fast developing tissues in the early stages of life, the demand on LC-PUFAs is especially high;

correspondingly, the expression from genes encoding desaturases and some other genes of the LC-PUFA biosynthetic pathway is high [10]. The precursors linoleic acid (LA) and α-linolenic acid (ALA) of ω6 and ω3 series LC-PUFAs, respectively, are essential. Products of their downstream conversions are generally considered as semi-essential. The endogenous pathway is considered to fulfil the function of a compensation apparatus under nutritionally fluctuating conditions.

The consensus LC-PUFA endogenous biosynthetic pathway involves alternating steps of desaturation (the introduction of an additional double bond inside the substrate polycarbon fatty acid chain) and elongation (the addition of two carbons), whereby in particular steps of the biosynthesis of the ω6 and ω3 series, substrates compete for the same enzymes. In the first and rate-limiting step, FADS2 converts C18:2 Δ9,12 (LA) and the C18:3 Δ9,12,15 (ALA) into C18:3 Δ6,9,12 (γ-linoleic acid, GLA) and C18:4 Δ6,9,12,15 (stearidonic acid, SDA), respectively. Downstream enzymatic steps in the biosynthesis are shown in Figure 1 [11]. Alternative Δ8 pathways are depicted as well, however, they remain less understood. The Δ8 pathway includes the initial elongation of essential precursors requiring the action of elongase (Elovl) enzymes. Elovl5 enzymes isolated from many fish species have been shown to possess elongation activity towards LA and ALA [12–15]. The elongation products C20:2 Δ11,14 (eicosadienoic acid, EDA) in ω6 LC-PUFA biosynthesis and C20:3 Δ11,14,17 (eicosatrienoic acid, ERA) in ω3 LC-PUFA biosynthesis undergo further desaturation by FADS2 enzyme acting, in this case, with Δ8 specificity [11,16].

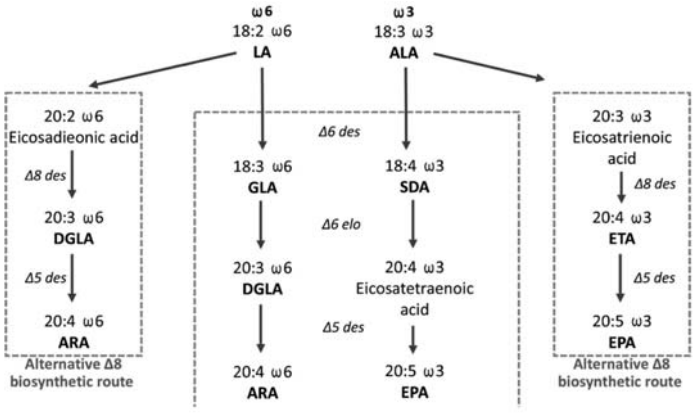

Figure 1. The scheme of the LC-PUFA endogenous biosynthesis of the ω6 and ω3 series from dietary C18:2 Δ9,12 (linoleic acid, LA) and C18:3 Δ9,12,15 (ALA) fatty acids. Dihomo γ-linolenic acid (DGLA): arachidonic acid (ARA): γ-linoleic acid (GLA): stearidonic acid (SDA): eicosapentaenoic acid (EPA): eicosatrienoic acid (ETA).

The developmental origins of the health and disease paradigm propose that, in the early stages of life, nutrition plays a critical role in establishing later-life health and susceptibility to diseases, as well as in some developmental disorders. It is crucial for embryonic success and for the long-term health of the organism that the yolk lipid content and utilization is not compromised [17]. Nonetheless, little is known about the influence of fatty acid composition on vertebrate embryogenesis and development. Recently, scientific reports transpired, which confirm the presence of active lipid biosynthetic processes in the first stages of life in which, among others, LC-PUFAs arise [18]; however, the significance of the endogenous LC-PUFA biosynthesis inside a yolk cell appreciates elucidation.

In humans, numerous observational studies have shown a link between childhood developmental disorders and LC-PUFA intake and ω6: ω3 LC-PUFA series imbalances,

such as some neurocognitive disorders or vision development [4,10,17]. Alterations in the cellular content of fatty acids can lead to the modulation of cellular signaling and eicosanoid production [19]. For ethical and practical reasons, however, the metabolic processes cannot be studied in the human embryo. Rodents, mainly mice, are typical model systems of a primary choice, which might not be the case in investigations regarding lipid metabolism for the following reasons.

Although rodents and humans are similar in many aspects of developmental biology and physiology, there are substantial differences between them in lipid metabolism [20,21]. Rodent embryos lack maternally supplied yolk as they survive in an environment of dynamic nutrition by being completely surrounded by metabolically active inverted yolk sac epitheliums. Hence, they do not totally rely on the bulk of nutrition provided by the mother. In contrast, human embryos have protruding yolk sacs that are more structurally similar to those occurring in some teleost fish, such as the popular model zebrafish. Their yolk sac contains the finite bulk of nutrients with which they sustain metabolic functions and growth, until the onset of placental–fetal exchange in humans or exogenous feeding in fish [21].

In addition, zebrafish exhibit features that have allowed them to gain steadily growing popularity in the lipid research community to use be used as the favorite model organism. Some of their popular features are the large number of offspring (one female zebrafish produces clutches containing 100 eggs on average), allowing for relatively robust sampling with synchronized external fertilization [22], followed by rapid development, and the simplicity and efficiency of genetic manipulations, such as the modern clustered regularly interspaced short palindromic repeat (CRISPR)-associated (CRISPR/Cas) system for genome editing [23,24]. Another feature is the optical transparency of the developing zebrafish, of which the relatively newly developed imaging techniques have taken advantage, enabling the direct visualization of lipids and uncovering the to date unseen lipid trafficking routes during the very early stages of life [18]. An insight into one single individual intact fish embryo has recently become possible by ambient ionization techniques, such as desorption electrospray ionization mass spectrometry and nano-electrospray mass spectrometry, enabling the direct analysis of lipids in individual zebrafish embryos [25]. Evidently, during the last few years, the insight into zebrafish lipid metabolism has significantly increased and become a powerful tool in lipid research, with implications of more diverse taxa, including humans.

Substrate specificities of FADS2 differ among fish species, and monofunctional and bifunctional desaturases with Δ6, Δ5, and Δ4 activities have been described [26]. The FADS2 capability of Δ8 activity has been described for both mammals [16] and fish [27]. Hence, two pathways are suggested to biosynthesize LC-PUFA EPA and ARA: the "classical" Δ6 pathway (Δ6 desatutation → elongation → Δ5 desaturation) and an alternative Δ8 pathway (elongation → Δ8 desaturation → Δ5 desaturation). In zebrafish, FADS2 represents the sole enzyme with desaturation activity towards PUFAs [28]. Using reverse genetics approaches, such as modern CRISPR/Cas9 technology, the zebrafish *fads2* gene represents the single target for manipulating desaturations.

Surprisingly, there is currently no study that studies fish *fads2* mutants in oocyte development. There is a vast amount of information available on the dietary lipid manipulation of brood stock allowing developing strategies for improving spawning performance and egg quality [29]. However, it is not clear if the role of the endogenous production of LA and ALA conversion downstream products is dispensable under the nutritionally rich conditions, hence replaceable by LC-PUFA biomolecules delivered by the diet, or the well-functioning endogenous LC-PUFA biosynthesis represents a prerequisite for reproduction health. Recently, a study appeared that confirmed the maternal transfer of *fads2* mRNA to the developing oocyte in gilthead seabream (*Sparus aurata*) [30]. We deduce that the eggs' fatty acid profile putative abnormalities are solely caused by the mother organism in fish. Using CRISPR/Cas9 technology, we determined the start codon of the *fads2* gene in zebrafish one-to-four-cell stage embryos, raised them until adulthood and determined the

degree of derived crispant mosaicisms. Female crispants displaying a degree of *fads2* gene editing higher than 50% were spawned. The eggs spawned by crispant females proceeded to lipid extraction, and the fatty acyl methyl ester preparations and LC-PUFA profiles were examined with special focus on the first steps of the biosynthesis of LC-PUFAs.

2. Materials and Methods

2.1. Ethics Statement

All experiments on the animals were conducted at the Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia in České Budějovice, Vodňany, Czech Republic. The facility has the competence to perform experiments on animals (Act no. 246/1992 Coll., ref. number 16OZ19179/2016-17214) in accordance with the principles based on the European Union Harmonized Animal Welfare Act of the Czech Republic.

2.2. sgRNA Design and sgRNA/CRISPR/Cas9 Complex Preparation

Our aim was to introduce mutations directly into the ATG start codon of the *fads2* gene to avoid the transcription of the FADS2 protein from the beginning. To achieve this, we used the CRISPR/Cas9 genome editing system consisting of endonuclease Cas9, which unwinds the genomic DNA duplex and creates double-strand breaks, and the single guide sgRNA, which is responsible for the site-specific recognition of the intended cleavage site. Prior to this, the synthetic sgRNA 5′-GTTCAGAGATCAGCGATGGG-3′ was designed according to the zebrafish *fads2* gene (zfin.org/ZDB-GENE-011212-1, accessed on 1 April 2022) and purchased from the SYNTHEGO company (Redwood City, CA, USA) (synthego.com, accessed on 1 April 2022); we made sure that sequences flanking the ATG start codon of our maintained zebrafish matched the sequence published on zfin.org. We PCR amplified the part of *fads2* gene using our PCR primers Fw 5′-TTTCCACCACGATCCACTGA-3′and Rev 5′-CAGTGGGTGGTGGTGGAG-3′, then gel-purified and sequenced (Macrogen) the amplicon. The Cas9 nuclease protein was freshly prepared before use, according to the manufacturer's protocol (Cas9 Nuclease Protein, Applied Biological Materials Inc. (Richmond, BC, Canada), abmgood.com/cas9-nuclease-protein-k008-vin.html, accessed on 1 April 2022).

2.3. Fish Source and In Vitro Fertilization

AB-line zebrafish (*D. rerio*), reproductively mature, were maintained in a zebrafish housing system (ZebTec®—Tecniplast system, Buguggiate, Varese, Italy) at a temperature of 28 °C and photoperiod 14L:10D, and fed twice daily with a standard diet (GEMMA Micro, http://www.zebrafish.skrettingusa.com, accessed on 1 April 2022). Prior to artificial fertilization, breeding pairs were transferred into spawning chambers in the afternoon before the spawning, whereby the male was separated from the female by a translucent plastic barrier. In the morning, the fish were anesthetized in a 0.05% tricaine solution (Ethyl3-aminobenzoate methane sulfonate). The stripped milt from the male zebrafish was examined for sperm motility under a Nikon SMZ745T stereomicroscope (Nikon, Tokyo, Japan). The pooled milt was added to an Eppendorf tube with 50 µL of an immobilizing solution (Kurokura 180 solution). The fish were transferred into fresh water for recovery. For the in vitro fertilization, the egg batches composed of approximately 100 ovulated oocytes were collected from the females by hand stripping and separately distributed into Petri dishes. Immediately, 10 µL of the pooled sperm and 200 µL of tank water was added, followed by gently shaking for about 45 s. Finally, the fertilized eggs of the control and treatment groups were gently distributed into Petri dishes.

2.4. Microinjection of the sgRNA/CRISPR/Cas9 Complex into the In Vitro Fertilized Eggs, and Fish Maintenance

All the components and sterile instruments were prepared to enable the rapid delivery of the sgRNA/CRISPR/Cas9 complex into the just-fertilized egg, ideally at the one-cell stage to minimize the obstacle providing CRISPR/Cas9—the mosaicism in the threated organism. Practically, we continued with microinjections until the 4-cell stage. Microinjec-

tions were performed as follows: approximately 50 eggs (one half of the batch following the fate of the samples) were injected. The final mixture was injected through the chorion inside each egg cell using the glass micropipette with a tip of 10 µm prepared from a glass needle (Drummond, Tokyo, Japan) by the puller (PC-10; Narishige, Tokyo, Japan). The manipulation with the full needle was achieved by the help of a hand manipulator under the microscope and under a pressure of 0.100 kPa/s developed by the injector. The eggs were maintained at 28 °C in the laboratory incubator. The unfertilized, undeveloped eggs and eggs missing the fluorescent signal due to the absence of 1% FITC-biotic-dextran, a confirmation of the introduced microinjection needle content, were continuously harvested and water was exchanged. The injected embryos were subsequently raised until sexual maturity, whereby, in the larval stage, they were transferred to ZebTec system for the genetically modified zebrafish.

2.5. Genomic DNA Isolation, the Detection and Quantification of the CRISPR/Cas9 System-Mediated Gene Editing

Genomic gDNA was extracted from fin clips, according to the manufacturer's protocol (Exgene^{TM}Genomic DNA micro, GeneAll®, Seoul, Korea) from each fish under anesthetization in 0.62 mM tricaine. Genomic regions flanking the CRISPR target codon start site was PCR amplified using a high-fidelity DNA polymerase. The sequences of the PCR primers are Fw 5′-TTTCCACCACGATCCACTGA-3′and Rev 5′-CAGTGGGTGGTGGTGGAG-3′. PCR products were electrophoretically separated in 1% agarose gel, followed by gel extraction (E.Z.N.A.® Gel extraction Kit, VWR, Radnor, PA, USA) and Sanger sequencing (Macrogen-Europe). DNA sequencing chromatograms were analyzed using the Inference of CRISPR Edits (ICE) free software tool (synthego.com/products/bioinformatics/crispr-analysis, accessed on 1 April 2022).

2.6. The Eggs' LC-PUFA FAMEs Profiles and Statistical Analyses

Ovulated eggs from wild-type and crispant females were collected in two repetitions (M1, M2) and sent to the customer service laboratory (BIOCEV, Charles University in Prague) for lipid extraction and the esterification into fatty acid methyl esters (FAMEs), followed by LC-PUFA analysis by comprehensive two-dimensional gas chromatography with a mass detector (GCxGC/MS). The FAME samples were prepared according to a previously used methodology [31]. Primary column Tr-FAME, 59 m, 250 µm ID, and 0.25 µm PT (Thermo, Waltham, MA, USA) was coupled to a secondary column Rxi-5MS, 1.2 m, ID—250 µm ID, 0.25 µm PT (Restek, Stockbridge, GA, USA). The temperature program was as follows: 90 °C (1 min); 10 °C/min; 140 °C (0 min); 4 °C/min; 200 °C (0 min); 10 °C/min; and 250 °C (6 min). The other parameters were set as follows: the flow was 1.2 mL/min; injection temperature was 240 °C; transfer line temperature was 280 °C; modulation period was 3 s; offset between the primary and secondary columns was 10 °C; and the hot-pulse time was 1.1 s. The mass detector was equipped with an Electron Ionization and a Time-Of-Flight analyzer. The temperature of an ion source was set to 280 °C. The analyzer scanning range was m/z 29–600. The data files were automatically processed in ChromaTOF software v4.7. FAMEs were identified comparing a two-dimensional retention behavior and mass spectra with commercial FAME standards (GLC 744, NU-CHEK-PREP) and mass spectra. The percentage distribution of LC-PUFA FAME signals in each sample of egg batch was counted.

The data were analyzed in R-studio software (R ver. 4.1.1) using default libraries and the pgirmess library.

The significance of the differences among the treatments in individual LC-PUFA ratios were tested using the parametric one-way ANOVA or non-parametric Kruskal–Wallis test, if the homoscedasticity assumptions were not fulfilled—this was tested by the Shapiro–Wilk normality test and Bartlett test of the homogeneity of variances ($\alpha = 0.05$). Multiple comparison tests were applied post hoc.

2.7. Phenotype of the Fertilized Eggs Produced by Crispant Females

Breeding pairs of reproductively mature wilt-type and crispant zebrafish (with a knockout score confirmed to be above 50%) were transferred from the housing system (ZebTec®—Tecniplast system, Buguggiate, Varese, Italy) into spawning chambers in the afternoon before the spawning fish (one male and one female) were separated from each other by a translucent plastic barrier. On the onset of light on the next day, the barrier was removed and the fish were observed for oviposition. A plastic mesh just above the bottom protected the eggs from being eaten by the parents. Following the spawning, the eggs were placed on Petri dishes and cultured at 28.5 °C in an incubator.

3. Results

3.1. sgRNA Design

The sgRNA sequence 5′-GUUCAGAGAUCAGCGAUGGG-3′ is the best hit, matching the requirements of being the early coding region of the gene targeted with minimum off targets and, at the same time, being a common exon and having a high activity, as shown in Figure 2.

Figure 2. The best hit of single guide RNA for targeting the ATG start codon of the *fads2* zebrafish gene (green box by Synthego).

3.2. The Detection and Quantification of the CRISPR/Cas9 System-Mediated Gene Editing

Mosaic founder mutant females of the G_0 generation were identified by genotyping. Sanger sequencing data files from the controls and each single crispant fish were delivered. We analyzed them using the free available software Inference of CRISPR Edits (ICE) tool, and calculated the overall editing efficiency and determined the profiles of all the different types of edits that are present and their relative abundances. A representative result is shown in Figure 3.

The relative contribution of each indel, percentage of indels, and mutagenesis efficiency (knockout score) were counted for each crispant fish. Only the crispants with the a knockout score above 50% were selected for further research and raised until adulthood

3.3. Egg Quality, LC-PUFA FAME Profile Analysis and Statistical Analysis

Egg sampling, storage, transportation, and manipulation has been optimized to obtain strong signals of prepared particular LC-PUFA FAMEs using the comprehensive two-dimensional gas chromatography. The percentage distribution of LC-PUFA FAMEs (particular LC-PUFA FAMEs correlated to the total PUFA FAMEs extracted) was used for the downstream statistics. All non-parametric tests of the differences among the treatments in individual response variables resulted significantly ($p < 0.05$; Table 1), except for C20:5, EPA. Post hoc multiple comparisons revealed that all the ratios were significantly different ($p < 0.05$) between the controls and the M1 or M2 groups (Figure 4).

Figure 3. The Inference of CRISPR Edits (ICE) software output of the analyses of the Sanger sequencing data on the *fads2* gene part flanking the mutated ATG start codon in fish representative (here, crispant female No. 1) (ice.synthego.com, accessed on 1 April 2022).

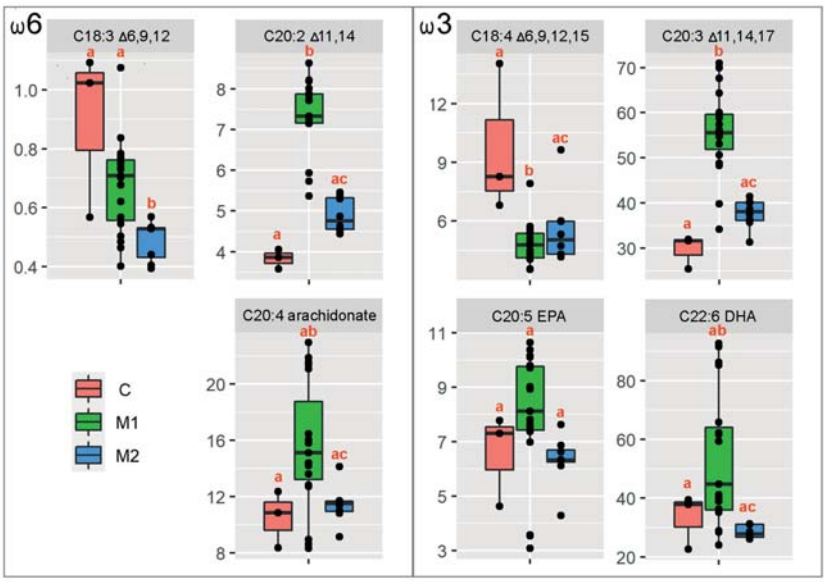

Figure 4. Box and whisker plots for the given LC-PUFA ratio summarizing the median value (black solid horizontal line), first and third quartiles, and minimum and maximum values. The different letters above particular box and whisker plots represent the significant statistical difference among the particular groups.

Table 1. The statistical results of the Kruskal–Wallis tests (*H*-value) and one-way ANOVA (*F*-value) for particular PUFAs.

	H-Value (*F*-Value)	Number of d.f.	*p*-Value
C18:3 Δ6,9,132	9.99	2	0.007
C20:2 Δ11,14	20.39	2	<0.001
C20:4, arachnidonate	9.37	2	0.009
C18:4 Δ6,9,12,15	7.56	2	0.023
C20:3 Δ11,14,17	17.50	2	<0.001
C20:5, EPA	2.12	2.27	0.139
C22:6, DHA	11.36	2	0.003

Our data demonstrated an increase of C20:2 Δ11,14 and decrease in GLA in the ω6 pathway, and an increase of C20:3 Δ11,14,17 and decrease in the SDA in ω3 pathway (Figure 5). This indicates the existence of a conversion of LA and ALA in the alternative Δ8 pathway of LC-PUFA biosynthesis in zebrafish partial *fads2* mutants. In comparison to the wild types, *fads2* mutants are significantly less able to desaturate LA and ALA in the Δ6 position.

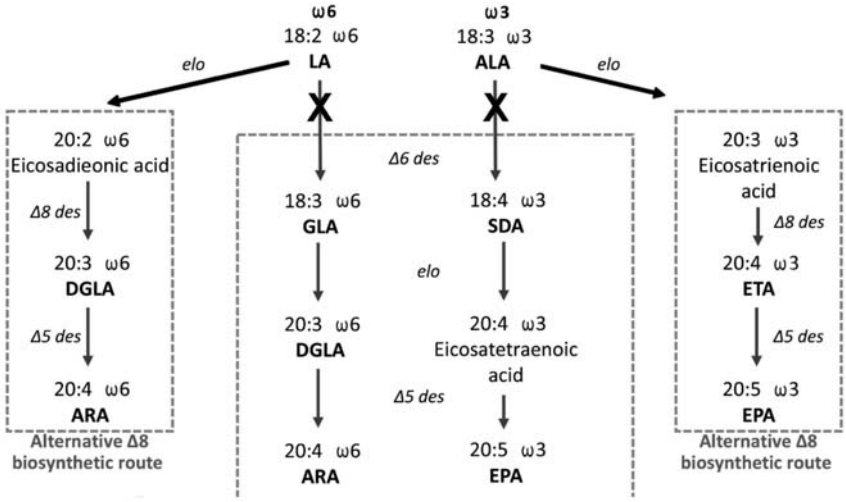

Figure 5. Scheme of the diversion of the LC-PUFA biosynthetic initial step of conversion of LA and ALA from the Δ6 pathway, rather than the Δ8 pathway in fads2 partial zebrafish mutants.

It is worth noting that AA, EPA, and DHA, generally considered as the most important polyunsaturates produced in LC-PUFA biosynthesis, did not significantly differ in their AA/LA and EPA/ALA and DHA/ALA ratios between the crispants and wild types. AA, EPA, and DHA are abundant in the artificial diet given ad libitum to fish. Nonetheless, even if the *fads2* gene depletion was not complete in our G_0 crispants (mutational rate: 50–80%) and their metabolism proved to maintain the production of main LC-PUFA biomolecules (bypassing the first desaturation step), the reproductive capabilities of females were influenced.

3.4. Altered Phenotype of the Embryos from a Fads2 Mutant Female x WT Male Inter-Crosses

The eggs fertilized successfully in the control WT female x WT male and crispant female x WT male inter-crosses were incubated in laboratory conditions at 28 °C for

approximately 24 h. Embryos obtained from crispant female x WT male inter-crosses were noticeably brownish in color and more densely packed in chorion, in comparison with the control WT when the embryos from both groups were aligned on the same Petri dish next to each other (Figure 6).

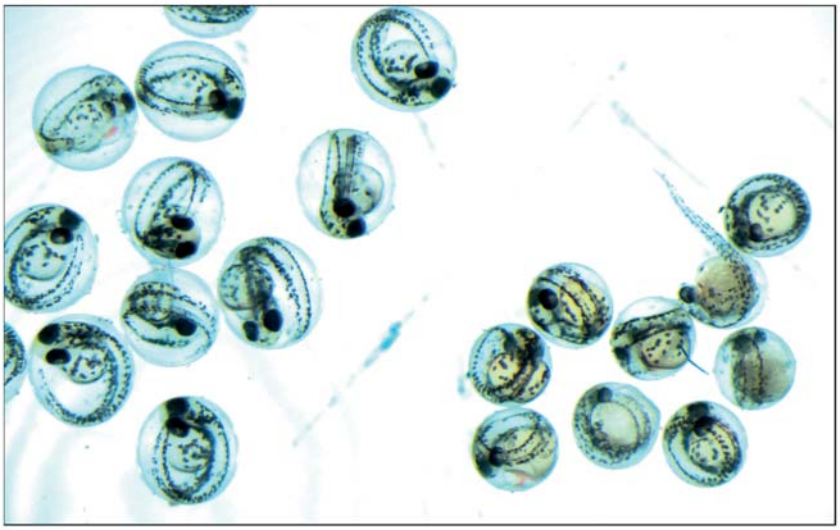

Figure 6. Embryonic lethality and affected development in embryos from crispant female x WT male inter-crosses (right side of the picture) and WT x WT (left side of the picture).

4. Discussion

In the present study, we investigated the functional link between the delta-6 desaturase gene (*fads2*) partial knockout and impaired reproductive success of zebrafish (*D. rerio*) females, whereby we observed that the *fads2* partial zebrafish knockout diverts from the "classical" LC-PUFA biosynthesis and converts C18 essential substrates LA and ALA via an alternative Δ8 pathway instead of the Δ6 desaturation pathway.

Based on the previous studies performed on mammals, infertility has been marked as a hallmark of FADS2 deficiency. It was confirmed that *fads2* mouse females are sterile [4,32]. Mammals are amniotic organisms and display substantial differences in reproductive functions, with regard to lipid metabolism from anamniotic organisms [20,21]. Indeed, to extrapolate lipid research conclusions between these diverse groups of organisms might be questionable, until a functional study of this phenomenon is conducted.

We used the popular model organism, zebrafish, applied the CRISPR/Cas9 genome editing tool to target the start codon of the *fads2* gene, raised generated potential crispants until adulthood, and then focused on the reproductive performances. Progenies of the crispant x crispant inter-crosses were not viable in our hands, and did not succeed to proceed to biallelic mutant generation. However, previous research confirmed that even if CRISPR-targeted G_0 generation fish were genotypically complex, the highly induced mutagenesis frequencies of somatic mutations could still result in obvious phenotypes in G_0 founders in zebrafish [23,33,34]. Taking the observations into account, we mated crispant females with wild-type males, with the aim to investigate a putative impact on egg quality. The oocyte development fully relies on the maternal nutritional supply, which consists of a large portion of lipids for which LC-PUFAs are of high importance. Zebrafish embryos have been confirmed to obtain *fads2* mRNA from the mother [30], hence the mother's lipid

metabolism is supposed to be responsible for the expected phenotypic prevalence of the *fads2* gene function.

In our study, we included zebrafish females, in which we determined the proportion of cells that have either a frameshift or 21+ bp indel (KO score) above 50%. To analyze his, we used the ICE software, for which the effectiveness has been rigorously evaluated by the Synthego company by analyzing thousands of edits performed over multiple experiments and comparing the robustness and accuracy [35,36]. The undeniable advantage of this tool for our study was that there was no need to euthanize the fish for the gene expression analysis to quantify the mRNA levels, which, at the same time, could be considered as a weakness of this study. Evidently, our study would greatly benefit from the transcriptomic analyses of zebrafish *fads2* partial knockouts.

Previous research has provided substantial evidence on the significant effects of dietarily obtained LC-PUFA levels and balance on brood stock reproductive success. However, the effect of LC-PUFAs with an endogenous LC-PUFA biosynthesis origin has been inadequately studied. Therefore, we fed zebrafish with a standard diet (GEMMA Micro, Westbrook, MA, USA. http://www.zebrafish.skrettingusa.com, accessed on 1 April 2022), with the aim to observe the phenotypes caused by impairments in the endogenous LC-PUFA biosynthesis, for which the fish itself cannot compensate, even if the diet is provided ad libitum. Endogenous production has been mainly hypothesized to serve as a compensation apparatus, which helps the organisms to maintain homeostasis under fluctuating environmental conditions and LC-PUFA availability. However, we hypothesized that endogenous biosynthesis performs functions that are not yet fully understood, and that our zebrafish *fads2* partial knockout model with a KO above 50% offers a unique insight into the organism that faces the situation of desaturation products not being completely depleted, yet significantly (without the depletion of essential LA and ALA at the same time) encourages the metabolism to undergo compensational measures, which are not known.

One important prerequisite for the present study is the absence of homologous genes encoded in zebrafish genomes, as well as the absence of functional splicing variants, both of these being investigated in the previous research. In zebrafish, only a single *fads* gene has been identified, which has been demonstrated to exhibit activity similar to that of both mammalian delta-6 and delta-5 desaturases [28,36]. Moreover, the zebrafish genome does not code a homologous gene to *fads3*, which could compensate for the Δ6 and/or Δ8 desaturation loss of function [37]. In *fads2*-null mice (−/−), no in vivo AA synthesis was detected after the administration of [$U^{13}C$] linoleic acid (LA), indicating the absence of the Δ6 desaturase isozyme [38]. Their study also showed the absence of the Δ6 desaturase isozyme for the first and last steps of desaturation in DHA and DPA ω6 LC-PUFA biosynthesis. Based on the current knowledge, we do not consider any Δ6 desaturation activity present in zebrafish performed with another enzyme. Theoretically, it should be considered that the targeting of the *fads2* gene start codon could cause the destruction of only one splicing variant by maintaining the possibility of the transcription of another splicing variant from an alternative start codon, which could be partly responsible for the presence of the activity of Δ8 desaturation. We do not consider that this occurred, based on the data delivered by Sibbons et al. [39] who excluded the alternative splicing of *fads2* gene.

According to our results, the fact that zebrafish face the situation of a significantly lower abundance of functional *fads2* genes makes decisions to keep the gene product to act only in those reactions, which are the most important for the maintenance of a healthy state. We observed that the mutants preferentially convert essential substrate LA and ALA via an alternative Δ8 pathway of LC-PUFA biosynthesis, bypassing the initial Δ6 desaturation step in which GLA and SDA are produced. It is probable that following the alternative Δ8 pathway saves more Δ6 desaturase enzyme molecules and maintains a higher capacity for downstream polyunsaturated products of LC-PUFA biosynthesis, such are the AA, EPA, and DHA. Hence, as a result of the improper functioning *fads2* gene, which cannot provide a sufficient amount of appropriate desaturation reactions,

the suffering zebrafish organism responds by bypassing the first conversion of essential LA and ALA precursors via the alternate route involving its elongation, followed by Δ8 desaturation instead of Δ6 desaturation followed by elongation. The mechanism of these reactions remains to be elucidated further. We speculate that a Δ6 desaturase activity cell deficient in Δ6 desaturase activity has a lower risk of losing GLA, when it might not be met with an appropriate elongase. Firstly, LA becomes subject to elongation in excess to obtain a higher probability for this molecule to meet with its FADS2 desaturase (performing desaturation in the Δ8 position, in this case). Even if the *fads2*-defective organism suffers from an inadequate GLA abundance, bypassing the first LA (and ALA) conversion, this seems to be an effort to rescue its conversion to a DGLA (and SA) molecule, which is an important substrate for not only other downstream omega-6 (and omega-3) LC-PUFAs, but also eicosanoids. On the other hand, the primary aim of the present study was to analyze zebrafish with an impaired function of the FADS2 enzyme, which supplies the organism by GLA by converting the essential LA. The content of GLA, unlike other LC-PUFAs of the n-6 series, is very low in food, hence the proper function of the FADS2 enzyme is crucial for the maintenance of the health of a vertebrate organism. Considering the fact that GLA serves precursors of prostaglandins and leukotrienes, we intended to answer the question of whether the endogenous LC-PUFA biosynthesis is indispensable in addition to the standard food conditions in which ω6 LC-PUFA biomolecules are normally delivered.

Recently, a study was conducted, which confirmed that salmon (*Salmo salar*) Δ6 FADS2 possesses Δ8 desaturation activities towards its substrates C20:2 d11, 14 and C20:3 d11, 14, 17 in vivo. In agreement with our observation, both substrates accumulate in *fads2* salmon knockouts [40]. Salmon has four *fads2* genes encoded in the genome Δ6fads2-a, Δ6fads2-b, Δ6fads2-c, Δ5fads2. The authors constructed two CRISPR-mediated partial knockouts Δ6*fads2*-abc/5 with mutations present in all four desaturases and knockout Δ6*fads2*-ab. Both knockouts displayed the degree of gene editing of 50–100%, which means that up to 50% of their desaturases might still work and/or compensate to offset each other. A genetic background that is too complex can enable the study of the milestone at which the organism can choose and divert to alternative routes to sustain a health-endangering situation. These authors suggested that the Δ8 desaturation pathway may be activated by high dietary levels of LA and ALA, and functions together with the Δ6 pathway to enhance the conversion of C18 precursors to downstream LC-PUFAs under limiting conditions. Our data indicate this scenario, since zebrafish partial *fads2* knockouts were able to produce AA, EPA, and DHA, generally considered as the most important products of LA and ALA conversions in LC-PUFA biosynthesis. Contrary to our expectations, AA, EPA, and DHA did not dramatically decrease. This could mean that the zebrafish body was capable of taking and store these biomolecules from the standard diet provided, and/or at the same time produce endogenously by the remaining LC-PUFA biosynthesis, something we did not intended to discern. The main conclusion of our observation is that not solely the abundance of AA, EPA, and DHA, or their relative ratios, counted towards the essential substrates AA/LA and EPA/ALA or DHA/EPA and the balance between ω6 and ω3 in fish, and, especially in brood stock, this should be considered in regard to state of health. Our study emphasizes the importance of the first step of LC-PUFA C18 substrates and suggests that the diversion through the Δ8 alternative pathway might at least be a sign of reproductive success impairments. Our study highlights partial *fads2* knockout zebrafish as a uniquely suited model organism, from which the investigation into disease pathogenesis, genetic diseases, and disorders caused by FADS2 function insufficiencies due to mutated *fads2* genes might considerably advance.

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CHAPTER 4

General discussion

Throughout the Earth´s history, the living organisms evolved to its current form in response to natural climate change, a slow natural phenomenon that has lasted for thousands or millions of years. However, in recent less than 100 years, anthropogenic greenhouse gases emission induced a rapid process of anthropogenic climate change.

The first publication of this dissertation thesis summarised the current knowledge and assessed fatty acid desaturase (FADS2) structure-function properties in fish in the context of environmental adaptations and as a target for genetic engineering. The work highlighted the FADS2 potential to play roles fundamental to adaptation to novel environmental conditions and reviewed investigations to elucidate the evolutionary history of fish FADS2, explaining the remarkable plasticity of this enzyme in fish, depicted the importance of FADS2 in the context of dwindling LC-PUFAs supply and mentioned a few remedies in the form of genetic engineering to improve endogenous LC-PUFA biosynthesis. If solutions in the form of genetically modified organisms come into practice, this will have a positive effect in sufficient delivery of health-promoting LC-PUFAs to animals and humans while at the same time preserving wild fish populations.

We have presented FADS2 as a fascinating enzyme with far-reaching implications for environmental sustainability and playing roles in adaptations to novel environments. At this place, it is worth mentioning the very interesting outcome from two publications showing that one single amino acid in FADS2 desaturase, when changed, switches the specificity towards substrates – the whole enzymatic function. Moreover, the study performed on *Manduca sexta* discovered that this one amino acid change led to considerable consequences in reproduction (Buček et al., 2015, 2020; Watanabe et al., 2016). Because FADS2 has the potential to switch the function in response to a single amino acid change with an impact on reproduction together with the predicted growing selection pressure forced by global warming in which, FADS has already been confirmed to play roles in fish (Ishikawa et al., 2019), we logically have asked: "How important is FADS2 for fish reproduction?"

Previous research has provided substantial evidence of significant effects of dietarily obtained LC-PUFA levels and ω3: ω6 LC-PUFA balance on broodstock reproduction success (Furuita et al., 2007; Callan et al., 2014; Turkmen et al., 2019; Sarih et al., 2020). The expression of FADS2 in blood of gilthead seabream (*Sparus aurata*) broodstock females was positively related to their reproductive performance in terms of fecundity and egg quality (Ferosekhan et al., 2020). However, the information on the impact of ablation of LC-PUFAs of endogenous origin due to *fads2* gene ablation, even under standard nutritional conditions, has been missing in fish and other anamniotic vertebrates as well.

In mice, the representative of amniotic vertebrates, *fads2* gene deletion mutants, both males and females, have been confirmed to be sterile (Stoffel et al, 2008; Stroud et al., 2009). Extrapolations from amniotes to anamniotes regarding aspects of lipid metabolism and its link to reproductive and developmental biology are questionable. The reason is the substantial differences in lipid metabolism (Miyares et al., 2014). Rodent embryos lack maternally supplied yolk as they are entirely surrounded by metabolically active inverted yolk sac epithelium (Ross and Boroviak, 2020). Hence, the nutrition they need is provided to them continuously. Anamniotes have the yolk sac with a finite bulk of nutrients provided by the mother and with which they have sustained metabolic functions and growth until the onset of exogenous feeding in fish or placental-fetal exchange in humans (Sant et al., 2018). That fact prompted us to fill this gap of knowledge. We asked more specifically: "Might the *fads2* gene with acquired mutations destroying its function allow the zebrafish (*Danio rerio*) to reproduce?"

We have chosen zebrafish for our model organism. Zebrafish has gained the steady growing popularity in the lipid research community to use it as the favourite model organism because

it exhibits features such are – a large number of offspring, allowing for robust sampling, rapid well documented development, simplicity and efficiency of genetic manipulations or optical transparency of the developing zebrafish enabling the direct visualisations (Westerfield, 2007; Jao et al., 2013; Varshney et al., 2015). For us, however, the far best feature of zebrafish and the main reason to choose it for our intended experiment was the zebrafish uniqueness in desaturation reactions in LC-PUFAs biosynthesis. All of them are performed by one single desaturase enzyme FADS2 encoded in the genome by a single *fads2* gene (Hastings et al., 2001). Zebrafish FADS2 was the first functionally characterised fish desaturase and has been confirmed to be a bifunctional enzyme catalysing desaturations in positions Δ6 as well as Δ5, hence called bifunctional. For some time, its bifunctionality has been considered as an exception, not only in fish but also in vertebrates in general (Monroig et al., 2009). Later, bifunctional desaturases were found in some other fish species such as the rabbitfish, the herbivorous marine teleost fish (*Siganus canaliculatus*) (Li et al., 2010), nile tilapia (*Oreochromis niloticus*) (Tanomman et al., 2013), pike silverside (*Chirostoma estor*) (Fonseca-Madrigal et al., 2014), carnivorous snakehead (*Channa striata*) (Kuah et al., 2016) and some others (Castro et al., 2016; Monroig et al., 2018). For us, zebrafish has represented an unprecedently suitable model organism with genetic background allowing target desaturations by mutating a single *fads2* gene to find out its functional link to reproduction.

According to the principle of reverse genetics, gene function can be defined by the inactivation of a given gene and analysis of the consequences. Targeted changes in the genome of living cells are powerful and fast-developing tools and the actively developing field of molecular biology. Recently, a genome editing tool clustered regularly interspaced short palindromic repeats-associated (CRISPR/Cas9) system was developed (Jinek et al., 2012). For CRISPR technology discovery, Jennifer Doudna and Emmanuelle Charpentier have been awarded the Nobel Prize in Chemistry in 2020. It is now the most popular system for genome editing because it is efficient and simple in use. It consists of endonuclease Cas9, which unwinds the genomic DNA duplex and creates double-strand breaks, and of single guide sgRNA, responsible for site-specific recognition of the intended cleavage site. The prerequisite for choosing a cleavage site is the presence of a protospacer adjacent motif (PAM) 5′-NGG-3′ (Chugunova et al., 2016). We aimed to design sgRNA to introduce mutations directly into the ATG start codon of the *fads2* gene. We successfully did this, as confirmed by the Sanger sequencing of the part of *fads2* gene flanking the start codon and its analysis by Inference of CRISPR Edits (ICE) software (https://www.synthego.com/products/bioinformatics/crispr-analysis) amplified from the genome of created zebrafish mutants and selected crispants displaying a degree of *fads2* editing within the range of 50–80%, the consequence of mosaicism phenomenon.

Using the CRISPR/Cas9 technology for generating gene-edited animals involves some obstacles. One such obstacle is mosaicism, which is common in founder animals (Yen et al., 2014). That is especially the case when the CRISPR/Cas9 system is used in embryos (Mehravar et al., 2019; Lamas-Toranzo et al., 2019), as we exactly did in our experiment with zebrafish. We have injected the CRISPR/Cas9 components as nucleic acid, and protein molecules into zebrafish fertilised zygotes. In essence, the CRISPR/Cas9 system could continuously target and cleave the gene at different stages of embryonic development, leading to mosaicism of the introduced mutations (Mizuno et al., 2014; Oliver et al., 2015), resulting finally in mosaic animals (Yen et al., 2014). Consequently, not all alleles inside the tissue or organism harbour mutations and mutations differ. They are called indels and are represented by insertions and deletions of different lengths. The presence of mosaicism complicates the phenotype analysis in F0 animals, and usually, it is suggested that both phenotype and genotype are analysed after breeding (i.e. at F1/F2 progeny) and upon obtaining homozygous mutant animals (Aslan et al., 2017). Mosaicism resulting from CRISPR/Cas9 genome editing in animal models is generally an undesirable outcome. However, this is not the case in our study. We have investigated the functional link between FADS2 and the reproductive success of zebrafish and have seen

mosaicism as an advantage which would allow to observe "mutated gene dosage effect" that might cause lethality and later in mutated (crispant) fish reproductive abnormalities. In our hands, partial *fads2* zebrafish knockouts displaying the degree of gene editing within the range of 50 to 80% were viable, and females produced egg batches. Eggs had a visibly yellow to brownish colour and tended to disintegrate. We confirmed differences in fatty acid lipid profile. According to our expectations, our data on fatty acyl methyl ester profiles on crispant female eggs when compared to wild type eggs confirmed the significant decrease of stearidonic acid (SDA, C18:4ω3), the Δ6 desaturation product from α-linolenic acid (ALA, C18:3ω3) and decrease of ɤ-linolenic acid (GLA, C18:3ω6) produced from linoleic acid (LA, C18:2ω6) in Δ6 desaturation catalysed by the same FADS2. However, we have not observed significant differences in the relative abundances of eicosapentaenoic acid (EPA, C20:5ω3) and arachidonic acid (ARA, C20:4ω6) downstream conversion products. Interestingly, the relative abundancies of eicosatrienoic acid (C20:3ω3) and eicosadienoic acid (C20:2ω6) were significantly higher in eggs produced by crispant when compared to the eggs produced by wild type females. That indicates that zebrafish, which faces the situation of depletion of a significant portion of functional *fads2* gene alleles, regulates the LC-PUFA biosynthesis by diverting the first conversion of essential precursors via an alternative route; instead of/beside the classical Δ6 pathway (Δ6 desaturation →elongation→ Δ5 desaturation) via the alternative Δ8 pathway (elongation → Δ8 desaturation → Δ5 desaturation). Similarly, salmon (*Salmo salar*) has been confirmed recently to possess Δ8 desaturation towards eicosadienoic acid and eicosatrienoic acid *in vivo*, which accumulated in *fads2* salmon partial knockouts (Datsomor et al., 2019). These authors suggested that the Δ8 desaturation pathway may be activated by high dietary levels of ALA and LA and functions together with the Δ6 desaturation pathway to enhance the conversion of C18 precursors to downstream LC-PUFAs under limiting conditions. An alternative pathway to LC-PUFAs activated under certain conditions has been hypothesized in mammals by Park et al. (2009), who confirmed FADS2 enzymatic activity in Δ8 position by functional characterization by heterologous expression in yeast. They concluded that Δ8 desaturation is a minor pathway, which might become important when there is high demand for eicosanoid synthesis, such as in inflammation or vasodilation. Δ8-desaturation by mediated direct conversion of eicosadienoic acid (20:2ω6) to dihomo- ɤ-linolenic acid (DGLA, 20:3ω6). Their findings provided unequivocal evidence for a novel alternative biosynthetic route to LC-PUFAs in mammals from substrates previously considered to be dead-end products. This alternative route to eicosanoid precursors might explain suggestions that eicosadienoic acid levels are related to human health. We suggest, Δ8-desaturation pathway is related to fish reproduction.

Going back to the assessment of FADS2 properties in fish regarding the environmental adaptations: Might the diverting or forking of LC-PUFA biosynthesis to the alternative Δ8 desaturation route be the solution how to better deal with the steady growing limitation of environmental conditions, then we suggest that the further research attention on this topic might focus on the first rate-limiting step and elucidate the molecular mechanisms standing behind the "making Δ8 – Δ6 decision". There is mounting evidence of species redistribution as the climate warms, and the very early initiation step of LC-PUFA biosynthesis might be under distinct evolutionary selection pressure. The higher ability of an organism to regulate the LC-PUFA biosynthesis and force the Δ8 desaturation pathway could predispose it not to be dyed out. Therefore, the future perspective of our work is to obtain transcriptomic data and determine the differently expressed genes between zebrafish embryos "walking classical Δ6 path" and embryos "walking alternative Δ8 path". The knowledge gained might possibly deliver new tips on transgenes to genetic engineers to enhance the LC-PUFA biomolecule production performance in some target organisms.

Conclucions

We conclude that FADS2 is a fascinating enzyme with far-reaching implications for human health, fish reproduction, and environmental sustainability. Obviously, FADS2 played an important role in adaptations to novel nutritionally pure environments throughout evolutionary history as documented across seawater and freshwater dwelling species and has excellent potential in the future, and to favour organisms to persist or colonise new ecological niches in times of global warming.

We confirmed the presence of an alternative Δ8 desaturation pathway (elongation → Δ8 desaturation → Δ5 desaturation) besides the classical Δ6 pathway (Δ6 desaturation → elongation → Δ5 desaturation) in zebrafish *in vivo*, which we explain as an active regulation of LC-PUFA biosynthesis under the depletion of functional *fads2* gene alleles following the CRISPR/Cas9 genome targeting. We suggest that zebrafish bifunctional Δ6/Δ5 desaturase is, in fact, the trifunctional Δ8/Δ6/Δ5 desaturase.

We have investigated the functional link between FADS2 and reproduction success of zebrafish females by observing the direct impact of partial *fads2* gene knock out on their egg batches fatty acyl methyl ester (FAMEs) profile.

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English summary

Throughout the Earth´s history, the living organisms evolved to its current form in response to natural climate change, a slow natural phenomenon that has lasted for thousands or millions of years. However, in recent less than 100 years, anthropogenic factors induced a rapid process of global warming. Nutritional quality, when defined as the value of the product for the consumer´s physical health, growth, development and reproduction, is at risk. Long-chain polyunsaturated fatty acid (LC-PUFA) biomolecules are essential dietary components for vertebrates including humans. However, LC-PUFAs provisioning by changing ecosystems is predicted to decline significantly. Animals and human are usually able to produce LC-PUFAs endogenously from shorter and less unsaturated eighteen carbon fatty acid precursors obtained from the diet. However, the yield of LC-PUFA biomolecules biosynthesized is often too low to meet physiological needs. Rather, LC-PUFA biosynthesis is considered to play a role as a compensational apparatus to maintain homeostasis under fluctuating environmental conditions. The first and rate-limiting step in LC-PUFA biosynthesis from the C18 essential precursors is being catalysed by FADS2 enzyme. It has been demonstrated that *fads2* was the key metabolic gene important for overcoming the nutritional constraints associated with colonisations of nutritionally pure water environments after glacial retreat. And even in human population, *fads2* showed strong signatures of selection in Greenland Eskimos colonising polar regions.

Chapter 2 of the thesis has summarised the current knowledge about the key enzyme FADS2 and assessed its structure-function properties in fish in the context of environmental adaptations and as a target for genetic engineering. The work highlighted the FADS2 potential to play roles fundamental to adaptations to novel environmental conditions and reviewed investigations to elucidate the evolutionary history of fish FADS2, explaining the remarkable plasticity of this enzyme in fish, depicted the importance of FADS2 in the context of dwindling LC-PUFAs supply and mentioned a few remedies in the form of genetic engineering to improve endogenous LC-PUFA biosynthesis. If solutions in the form of genetically modified organisms come into practice, this could have a positive effect in sufficient delivery of health-promoting LC-PUFAs to animals and humans while at the same time preserving wild fish populations.

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Previous research has provided substantial evidence of significant effects of dietarily obtained LC-PUFA levels and ω3:ω6 LC-PUFA balance on broodstock reproduction success. However, the information on the impact of ablation of LC-PUFAs of endogenous origin was missing in fish and other anamniotic vertebrates as well. That fact prompted us to fill this gap of knowledge. Chapter 3 of this thesis investigated a direct functional link between *fads2* gene and reproduction success in zebrafish (*Danio rerio*) by reverse genetic approach – CRISPR/Cas9. We have investigated the functional link between FADS2 and reproduction success of zebrafish females by observing the direct impact of partial *fads2* gene knock out on their egg batches fatty acyl methyl ester (FAMEs) profile. We observed the presence of an alternative Δ8 desaturation pathway (elongation → Δ8 desaturation → Δ5 desaturation) besides the classical Δ6 pathway (Δ6 desaturation → elongation → Δ5 desaturation) in zebrafish *in vivo*, which we explain as an active regulation of LC-PUFA biosynthesis under the depletion of functional *fads2* gene alleles following the CRISPR/Cas9 genome targeting. We suggest that zebrafish bifunctional Δ6/Δ5 desaturase is, in fact, the trifunctional Δ8/Δ6/Δ5 desaturase.

Czech summary

V průběhu historie Země se živé organizmy vyvíjely v reakci na přirozenou změnu klimatu, pomalý přírodní jev trvající tisíce až miliony let. V posledních necelých 100 letech však antropogenní faktory vyvolaly rychlý globální proces oteplování. Následkem toho nutriční kvalita, pokud ji definujeme jako hodnotu produktu pro fyzické zdraví, růst, vývoj a reprodukci spotřebitele, začíná klesat. Jedním z hlavních důvodů je nižší zastoupení jedněch z esenciálních složek potravy pro člověka a prakticky všechna zvířata – biomolekul vysoce nenasycených mastných kyselin s dlouhým řetězcem (LC-PUFAs, z angl. *long-chain polyunsaturated fatty acids*). Obratlovci obvykle mají schopnost tvořit si LC-PUFAs uvnitř svých buněk z esenciálních kratších a méně nenasycených osmnáctiuhlíkových mastných kyselin, prekurzorů, které přijímají ve stravě. Ovšem tato schopnost často nestačí pokrýt fyziologické potřeby organizmu. Spíše se předpokládá, že endogenní dráha biosyntézy LC-PUFAs plní funkci jakéhosi kompenzačního aparátu, který pomáhá organizmu udržet si homeostázu za kolísajících podmínek prostředí. První krok biosyntézy je katalyzován enzymem FADS2, který je v genomu kódován genem *fads2*. Bylo prokázáno, že tento gen v minulosti sehrál klíčovou roli, kdy některým rybám umožnil kolonizovat nutričně chudé prostředí sladkých vod po ústupu ledovců. Dokonce i v lidské populaci vykazoval *fads2* gen silné známky selekce u grónských Eskymáků osídlujících polární oblasti, protože jim umožnil odpovídat na nové podmínky prostředí tvorbou LC-PUFA biomolekul.

Kapitola 2 zdůraznila potenciál FADS2 hrát zásadní roli v přizpůsobování se novým podmínkám prostředí a zhodnotila dosavadní výzkum v této oblasti s cílem objasnit evoluční historii FADS2 u ryb, přičemž vyzdvihla pozoruhodnou plasticitu FADS2. Nastínila i některá řešení genetického inženýrství v podobě geneticky modifikovaných organizmů. Taková řešení, byla-li by uvedena do praxe, by mohla mít pozitivní efekt na dodávání LC-PUFAs při současném zachování populace divokých ryb. Prezentovali jsme FADS2 jako fascinující enzym s dalekosáhlými důsledky pro udržitelnost životního prostředí. Z dosavadního výzkumu jasně plyne, že množství a složení jednotlivých LC-PUFAs přijímaných v potravě má významný vliv na reprodukční vlastnosti generačních ryb. Ovšem dosud nebyly provedeny studie, které by se zabývaly vlivem delece genu *fads2* a následným úbytkem endogenně vytvořených LC-PUFA biomolekul na reprodukci ryb, a dokonce ani anamniot obecně.

Kapitola 3 zkoumala reverzně genetickým přístupem přímou funkční souvislost mezi mutacemi v genu *fads2* a některými vlastnostmi souvisejícími s reprodukcí u vhodného modelového organizmu ryby – zebřičky (*Danio rerio*). Pomocí metody cíleného editování genomu CRISPR/Cas9 jsme připravili mutanty zebřiček nesoucí mutace v oblasti začátku *fads2* genu. Byly dochovány do dospělosti a poté byly vytřeny. U získaných jiker byl stanoven profil mastných kyselin. Bylo dokázáno, že u nich existuje alternativní Δ8 desaturační dráha (elongace → Δ8 desaturace → Δ5 desaturace) spolu s klasickou Δ6 dráhou (Δ6 desaturace → elongace→ Δ5 desaturace) *in vivo*. Vysvětlujeme si ji jako aktivní regulaci biosyntézy LC-PUFAs za podmínek nedostatku funkčních alel *fads2* genu. Naše výsledky ukazují, že bifunkční desaturáza Δ6/Δ5 je ve skutečnosti trifunkční Δ8/Δ6/Δ5 desaturáza.

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- Grant Agency of the University of South Bohemia in České Budějovice (GAJU 097/2019/ZBlahova)

List of publications

Peer-reviewed journals with IF

Bláhová, Z., Franěk, R., Let, M., Bláha, M., Pšenička, M., Mráz, J., 2022. Partial *fads2* knockout diverts LC-PUFA biosynthesis via an alternative Δ8 pathway with an impact on the reproduction of female zebrafish (*Danio rerio*). Genes 13: 700. (IF 2021 = 4.141)

Bláhová, Z., Harvey, T.N., Pšenička, M., Mráz, J., 2020**.** Assessments of fatty acid desaturase (Fads2) structure-funaction properties in fish in the context of environmental adaptations and as a target for genetic engineering. Biomolecules 10: 206. (IF 2020 = 4.897)

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Training and supervision plan during study

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Ph.D. courses	**Year**
Biostatistics	2018
Pond aquaculture	2018
Applied hydrobiology	2018
Ichthyology and fish taxonomy	2020
English language	2020

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2017 2018 2019 2020

International conferences	**Year**
Bláhová, Z., Baloch, A.R., Franěk, R., Pšenička, M., Mráz, J., 2017. Knocking out of delta-6 desaturase by CRISPR/Cas9 in common carp (*Cyprinus carpio L.*). 15th Euro Fed Lipid Condgress: Oil, Fats and Lipids: New Technologies and Applications for a Healthier Life. 27–30 August 2017, Uppsala, Sweden.	2017

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Centre for Integrative Genetics, Norwegian University of Life Sciences, Norway, (1 month, performing precision-cut liver slice culture tool to study fish lipid metabolism and cell responses *ex vivo*).	2017

Pedagogical activities	**Year**
Leading of project entitled ´How to create fish mutants in the "New era of molecular biology"? – CRISPR/Cas9 – mediated genome editing in zebrafish (*Danio rerio*)´ at International summer school.	2019

Curriculum vitae

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2000–2005 Dipl.-Ing., Faculty of Agriculture, University of South Bohemia, České Budějovice, Czech Republic; Master Thesis "Influence of genetically modified organisms on non target organisms"

PROFESSIONAL EXPERIENCE

2022 – present Technician in Laboratory of Aquatic Toxicology and Ichtyopathology, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, Czech Republic

2021 – present Project manager, Biology Center Czech Academy of Sciences, České Budějovice, Czech Republic

2020–2021 SARS-CoV2 diagnostic, Department of Medical Biology, Institute of Chemistry, University of South Bohemia, České Budějovice, Czech Republic

2016–2020 Worker in fisheries, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, Czech Republic

2005–2015 Worker in biological sciences, Czech Academy of Sciences and University of South Bohemia, České Budějovice, Czech Republic

TRAINING

Certificate of professional competence for designing experiments and experimental projects under Section 15d (3) of Act No 246/1992 Coll., on the Protection of Animals against Cruelty, as amended, First Faculty of medicine, Charles University, Prague, Czech Republic

Certificate of participation in the training course "Manipulations with hazardous chemical substances and mixtures"

RESEARCH STAYS

2017 1 month stay at Centre of Integratice Genetics, Norwegian University of Life Sciences, Norsko

2008 1 month stay at University of California Riverside, Los Angeles, USA