# Lipid Quality of Common Carp (*Cyprinus carpio*) in Pond Culture

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Cover: Selection of carps for an experiment
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## Abstract

There is a large body of evidence that n-3 fatty acids, especially EPA and DHA are beneficial for human health. Content of these fatty acids in fish fillet can be influenced by several factors.

This thesis examined differences in lipid content, lipid class and fatty acid composition among three parts of common carp fillets. The effect of genetic background on lipid content and fatty acid composition in common carp was investigated. An approach of using biologically active compound sesamin which might modulate the fish metabolism to synthesize more n-3 HUFA from ALA was tested.

The results showed that lipid content and composition differed strongly among the three parts of common carp fillet. The lowest lipid content was found in dorsal white muscle (0.95±0.14%), medium in red muscle (16.7±5.0%), whereas the highest was found in abdominal wall (30.2±7.8%). Abdominal wall with the highest lipid content was dominated by triacylglycerols whereas the white muscle had the highest contribution of phospholipids. The total lipid fatty acid composition differed greatly depending on the lipid content and ratio between phospholipids and triacylglycerols. The fatty acid composition of the leanest part, the white muscle, contained a large proportion of n-3 highly unsaturated fatty acids and a ratio n-3/n-6 = 1.1, having a high proportion of phospholipids. The abdominal wall was rich in monounsaturated FA and had a lower ratio n-3/n-6 = 0.5.

There were only slight differences in muscle fatty acid composition among four carp crossbreeds caused probably by differences in lipid content. Addition of sesamin did not alter muscle lipid composition in common carp.



*Keywords:* common carp, crossbreeds, fatty acid composition, fillet parts, nutrition, pond culture, sesamin.



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# Contents

# List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Mráz, J., Pickova, J. (2009). Differences between lipid content and composition of different parts of fillets from crossbred farmed carp (*Cyprinus carpio*). *Fish Physiology and Biochemistry* 35, 615-623.

II Mráz, J., Schlechtriem, Ch., Olohan, L.A., Fang, Y., Cossins, A.R., Zlabek, V., Pickova, J. (2010). Sesamin as a potential modulator of fatty acid composition in common carp (*Cyprinus carpio*). *Aquaculture Research* 41, e851-e861.

The contribution of Jan Mráz to the papers included in this thesis was as follows:

I Carried out the lipid analyses, processed the data and was responsible for compiling the manuscript.

II Performed the experiment with fish, collected the samples, carried out the lipid analyses, processed the data and was responsible for compiling the manuscript.

# Abbreviations

| | |
|---|---|
| AA | Arachidonic acid |
| ALA | Alpha-linolenic acid |
| CYP | Cytochrome P450 |
| Δ | Delta |
| DHA | Docosahexaenoic acid |
| EPA | Eicosapentaenoic acid |
| EROD | Ethoxyresorufin O-deethylase |
| FA | Fatty acids |
| FAME | Fatty acid methyl esters |
| GC | Gas chromatography |
| HUFA | Highly unsaturated fatty acids |
| HPLC | High performance liquid chromatography |
| LA | Linoleic acid |
| MUFA | Monounsaturated fatty acids |
| PL | Phospholipids |
| PUFA | Polyunsaturated fatty acids |
| SFA | Saturated fatty acids |
| TAG | Triacylglycerols |
| TLC | Thin layer chromatography |

# 1 Introduction

## 1.1 Common carp

Common carp (*Cyprinus carpio*) is one of the most cultured fish in the world. In 2008, the world and the European production was 2 987 433 tons and 144 747 tons, respectively (FAO, 2011). It is a well established cultured species with a well known production cycle. It is consumed as a traditional food in central Europe. Carp is an omnivorous species eating plankton and benthos (worms, insects, molluscs) as well as detritus in the natural conditions (Adamek *et al.*, 2004a). Typical way of farming is using artificial shallow earthen ponds in which the production is based on plankton and benthos production supplemented by cereals. The carp's digestive system is adapted to a diet including more carbohydrates compared with carnivorous species. The farming cycle in Europe usually takes 3-4 years.

Common carp is divided into two subspecies, *C. c. carpio* from Europe and *C. c. haematopterus* from Asia. Productive populations were domesticated from both ancestral forms, as well as their hybrids and backcrosses followed by mass selection (Vandeputte, 2003).

## 1.2 Fatty acids and aquaculture

The world capture fisheries have been relatively stable in the past decade and are predicted to be relatively stable in the future. It is not possible to further increase the fish capture in world-wide scale otherwise there would be a problem with overfishing and potential depletion of resources. The aquaculture production is the only solution to meet the increasing consumer demand for fish. Aquaculture is the fastest growing animal-food-producing sector with a growth rate from 1970 of around 8.3% per year and with 52.5

million tons (in 2008; 68.3 including aquatic plants) accounts for almost half of total food fish supply (SOFIA, 2010).

Fish oil and fish meal have been traditionally used as the basal ingredients in aqua-feeds for carnivorous fish culture. Fish oil has a high level of the n-3 highly unsaturated fatty acids (n-3 HUFA; carbon chain length $\geq$ C20 with $\geq$ 3 double bonds), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are healthy for fish as well as for humans. Since the aquaculture expands, fish meal and fish oil become more expensive. It consequently creates a high pressure on the aqua-feed producers to replace these ingredients with more sustainable alternatives (Pickova & Morkore, 2007). Generally, the vegetable sources of oil and protein are used as "The replacement". The vegetable oil can replace substantial amount of fish oil in the diets of many fish species without affecting growth and feed efficiency. However, the drawback of these alternatives is that they lack the n-3 HUFA and therefore are compromising the nutritive value of farmed fish for consumers. Several alternative oil sources, derived from unicellular algae, pelagic organisms or benthic invertebrates containing high amounts of n-3 HUFA have been identified and tested in aquafeeds. Nevertheless, their prices are still too high to be commonly used in aquafeeds (reviewed by Turchini *et al.* (2009)).

Carps represent the largest group of cultured fish constituting around 70% of freshwater aquaculture production. In Europe, the majority of carp production takes place in central Europe where it is produced in ponds using traditional semi-intensive techniques. There are two sources of n-3 HUFA in carp produced in ponds. The one is the natural feed, plankton and benthos, which is rich in n-3 HUFA and the other is the n-3 HUFA synthesized by carp from alpha linolenic acid (ALA). It was reported that carps, in contrast to marine fish, are be able to bio-convert ALA to EPA and DHA (Zheng *et al.*, 2004; Tocher, 2003; Olsen *et al.*, 1990; Farkas, 1984). It is therefore of interest to understand and maximize the ability of carp to synthesize EPA and DHA from ALA in order to preserve the lipid quality of the fish as human food and for sustainable utilization of feed resources. Carp culture might be capable of becoming net producer of EPA and DHA by selecting fish with high enzyme activities in fatty acid elongation and desaturation.

The carps have also relatively low requirements both for n-3 and n-6 fatty acids (0.5-1%) which can be fulfilled by plant 18 carbon fatty acids (Takeuchi, 1996). Inclusion of fish meal (5%) and fish oil (0%) in carp culture is also very low (Tacon & Metian, 2008) and therefore the

substitution of fish meal and oil will be considerably easier than for carnivorous aquaculture.

## 1.3 Fatty acids and their metabolism

Fatty acid is a carboxylic acid with aliphatic chain which could be either saturated (without double bonds) or unsaturated (with double bonds). Unsaturated fatty acids are further divided according to number of double bonds into monounsaturated (one double bond) or polyunsaturated (more than one double bond). The double bond can be organised in cis or trans configuration. Another important characteristic is a position of the first double bond (from the methyl end).

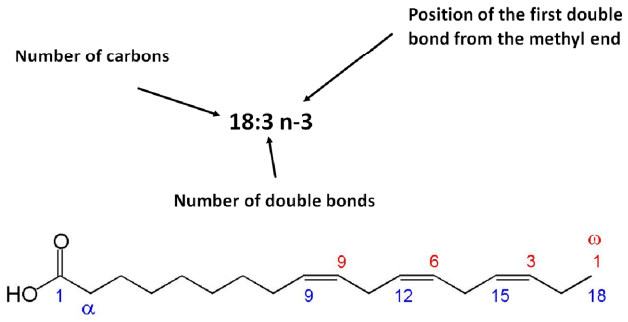


*Figure 1.* Principle of fatty acid nomenclature

Fatty acids are an important source of energy. In the form of triacylglycerols, they can yield more than twice the amount of energy for the same mass as carbohydrates or proteins do. In the form of phospholipids, they serve as a basic building block for all the cellular membranes. The fatty acid metabolism consist of catabolic processes which generate energy and fatty acid metabolites, and anabolic processes which lead to creating fatty acids and other molecules which they are part of.

Fatty acids are predominantly formed in liver from two-carbon body - acetyl-CoA through action of cytosolic multienzyme complex called fatty acid synthetase. All the known organisms are able to biosynthesize *de novo* saturated fatty acids. The saturated fatty acids can be further modified by inserting a double bond by Δ9 desaturase which is located in endoplasmatic reticulum. There are two series of polyunsaturated fatty acids (PUFA) which

cannot be formed by all vertebrates (including fish) and are essential for them. The n-6 family is based on linoleic acid (18:2n-6; LA) and the n-3 family is based on alpha-linolenic acid (18:3n-3; ALA). These two 18 carbon fatty acids can be further converted to highly unsaturated fatty acids (HUFA) by desaturases and elongases (Fig. 2) (Zheng *et al.*, 2004).

The rate of the conversion varies in different organisms usually depending on the extent to which the species can obtain HUFA from the natural diet. Thus the carnivorous species which can obtain excess of HUFA from natural diet have usually lower ability of conversion compared with herbivorous species. In marine fish species this bioconversion occurs poorly if at all and therefore they have essential requirements for HUFA in their diets (reviewed by Tocher (2003)).

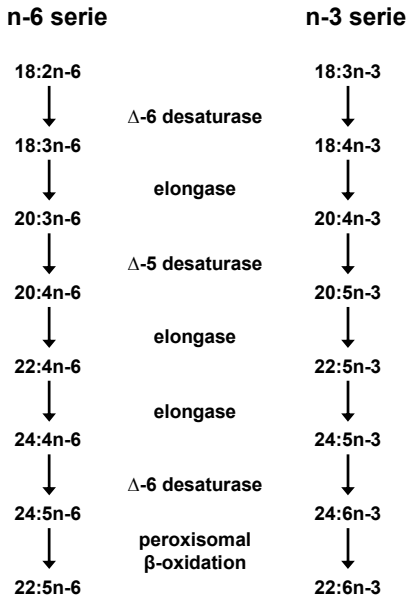


*Figure 2.* Pathways of HUFA biosynthesis from C18 fatty acids, adapted from Voss *et al.* (1991)

## 1.4 Fatty acids and human health

There is a large body of evidence that n-3 highly unsaturated fatty acids, especially EPA and DHA are beneficial for human health. EPA and DHA have many different functions and actions in human body, e.g.: influencing the physical nature of cell membranes, membrane-protein-mediated responses, being eicosanoid precursors, cell signaling and gene expression in many different cell types (Calder & Yaqoob, 2009). EPA and DHA have been shown to have beneficial effect in a range of cardiovascular risk factors, which result in primary cardiovascular prevention, reduction in total and cardiovascular mortality (Calder & Yaqoob, 2010).

Several studies indicate that conversion of ALA to EPA occurs but it is limited in humans and that further transformation to DHA is very low (Burdge & Calder, 2005). Therefore it was proposed that EPA and DHA should be consumed directly to maintain optimal tissue functions.

Today's western diet is generally deficient in n-3 fatty acids and excessive in n-6 resulting in a low n-3/n-6 ratio. It was proposed that human being evolved on a diet with the n-3/n-6 ratio close to 1 whereas in the western diet it exceeds 1:15 (Simopoulos, 2008; Leaf & Weber, 1987). This dietary change is associated with pathogenesis of many diseases, including cardiovascular diseases, cancer, inflammatory and autoimmune diseases.

Beneficial effect of EPA and DHA is generally recognized, however, there have been several concerns about safety of fish consumption mainly due to potential hazard of contaminants (mercury, PCB, dioxines). Mozaffarian & Rimm (2006) conducted an extensive meta-study and concluded that the benefits of fish intake exceed the potential risks of possible harmful effect of pollutants.

Many leading authorities and nutrition and health organizations have developed specific dietary recommendations for n-3 fatty acids and fish intake for different countries around the world. The European Food Safety Authority (EFSA) approved several health claims related to the consumption of fish or EPA and DHA, e.g., maintenance of normal level of blood triacylglycerols, normal brain function and vision, cardiac function and blood pressure (EFSA Panel on Dietetic Products, 2010). EFSA also proposed reference labeling intake values for general population: 250 mg EPA+DHA; 2 g ALA and 10 g of LA per day (EFSA, 2009). Recommended fish intake was set to 1-2 servings of oily fish per week. WHO/FAO (2003) recommended that regular fish consumption (1-2 servings per week) is protective against coronary hearth diseases and ischemic stroke (the serving should contain 200-500 mg EPA+DHA). The American Heart Association recommends for general population to eat

variety of (preferably fatty) fish at least twice a week (Kris-Etherton *et al.*, 2002).

Fish intake is generally low in the Czech Republic (in 2008: only 5.5 kg of fish or fish products per capita per year (MZe, 2009)) and it is far below current recommendations. Other sources of n-3 FA, including EPA and DHA, are scarce in diet consumed by Czech population (Hibbeln *et al.*, 2006). Thus, it would be beneficial to generally increase fish consumption and also to increase content of the beneficial fatty acids in locally produced fish and other products.

## 1.5 Factors influencing lipid content and composition

### 1.5.1 Species

Species of the animal is the first factor which influences lipid content and composition. It is easier to influence the FA composition in monogastric animals than ruminants. The reason is that unsaturated FA are hydrogenated by microorganisms in the rumen. Therefore ruminants have usually lower level of PUFA in the flesh lipids than monogastric animals.

There are huge differences in muscle lipid content among fish species which also lead to differences in FA composition (Fontagne 2010). Some fish species have very low lipid content in fillet (less tan 2%), e.g., pikeperch (*Stizostedion lucioperca*), European perch (*Perca fluviatilis*), and Atlantic cod (*Gadus morhua*). On the other hand some fish species can have very high lipid content in the fillet (more than 10%), e.g., Atlantic salmon (*Salmo salar*), European eel (*Anguilla anguilla*) (Henderson & Tocher, 1987). Generally, lipids of marine fish species contain more highly unsaturated fatty acids with higher n-3/n-6 ratio than the freshwater fish species (Henderson & Tocher, 1987).

### 1.5.2 Nutrition

"You are what you eat" so the nutrition has a major impact on the lipid content and composition in monogastric species. Fish reflect the lipid pattern of its diet to a high extent. Carp is traditionally reared in earthen ponds and its nutrition is based on natural food with cereal supplementation (Buchtova et al., 2007).

The natural food consists mainly of zooplankton, zoobenthos and detritus (Adamek *et al.*, 2004b; Adamek *et al.*, 2003). Plankton (Domaizon et al., 2000) and benthos (Bell *et al.*, 1994); (Bogut *et al.*, 2007) naturally contain high levels of n-3 FA, including EPA and DHA. Thus a proper pond

management maintaining sufficient amount and appropriate structure of planktonic and benthic community is of great importance when improving carp fatty acid composition.

Cereals are usually used as a supplemental feeding for carp. Since they are rich in carbohydrates and have very low level of n-3 fatty acids, the flesh of the farmed carps generally contains a high level of oleic acid and low level of favorable n-3 HUFA (Csengeri, 1996b).

Carp was observed to have the ability for n-3 HUFA biosynthesis from its precursor ALA (Tocher, 2003). Thus supplemental feeding which is rich in ALA could be the alternative way to increase n-3 HUFA content in carp flesh. Feeds with a high level of ALA, cheap and easily available, are rapeseed, linseed and hempseed. Rapeseed or rapeseed cake is becoming an important part of pellets for carp nutrition in the Czech Republic for its low price and availability. Rapeseed oil has a moderate amount of ALA (13%) and favorable ratio n-3/n-6 around 1:2 (Pickova & Morkore, 2007) and is commonly used in feed for salmonids as a replacement for fish oil (Bell *et al.*, 2001). However, there are no available data about impact of rapeseed oil on carp lipid content and composition when being used in pond production systems where many factors influence the muscle lipid composition, such as variations in amount and composition of natural food, fish density or environmental conditions as well as interactions among them.

### 1.5.3 Bioactive compounds

An alternative approach to influence muscle lipid composition might be the use of biologically active compounds which modulate the fish metabolism to synthesize or deposit more n-3 HUFA.

Such a potent compound could be sesamin. As the first study investigating sesamin effects in fish Trattner *et al.* (2008a) found that sesamin/episesamin supplementation increases the level of DHA up to 37% in white muscle of rainbow trout (*Oncorhynchus mykiss*) fed by high ALA vegetable oil. An *in vitro* study with Atlantic salmon (*Salmo salar*) hepatocytes Trattner *et al.* (2008b) showed that sesamin/episesamin exposure led to increased elongation and desaturation of $^{14}C$ ALA to DHA indicating that sesamin has modulatory effects on lipid metabolism leading to increased levels of DHA and higher β oxidation activity. However, there are still many questions about the use of sesamin in fish feed, especially whether effects similar to those observed in salmonids can also be observed across different fish species, particularly cyprinids.

Another potential bioactive compound might be lipoic acid. Lipoic acid acts as an antioxidant both in the hydrophilic and hydrophobic phases

(Navari-Izzo *et al.*, 2002). It was reported to have several effects on lipid metabolism in chicken (Hamano, 2006) and rats (Mythili *et al.*, 2006). Trattner *et al.*, (2007) studied effect of lipoic acid on fatty acid composition in brain and muscle in South American pacu (*Piaractus mesopotamicus*) and found that lipoic acid increased level of EPA in muscle polar lipids.

Conjugated linoleic acid and tetradecylthioacetic acid were also proposed to have stimulatory effect on DHA synthesis in salmonids (Kennedy *et al.*, 2007).

### 1.5.4 Genetic background

Another important factor affecting lipid content and composition is genetic background. It was shown that muscle lipid content is a highly heritable trait (>0.5) in common carp and that there is a relatively high positive genetic correlation between body size (standard length and body weight) and lipid content (0.71 and 0.59, respectively) (Kocour *et al.*, 2007).

There is evidence from mammals and birds that there is a heritable genetic component governing capacity to biosynthesize and/or deposit n-3 HUFA (Khang *et al.*, 2007; Karamichou *et al.*, 2006; De Smet *et al.*, 2004).

Leaver *et al.*, (2011) analyzed flesh lipid parameters in 48 families of Atlantic salmon and showed that flesh n-3 HUFA composition is a highly heritable trait ($h^2 = 0.77 \pm 0.14$). Eight families were further selected for transcriptomic analyses. They found that there were specific hepatic mRNA expression patterns associated with high flesh n-3 HUFA, which indicate possible mechanisms for family-dependent deposition in flesh.

There are no relevant data available for carp about the effect of genetic origin on the lipid composition (Fauconneau *et al.*, 1995). In a study with four carp hybrids Buchtova *et al.* (2007) found that, the fatty acid composition was not affected to any great extent by the hybrid type.

### 1.5.5 Sex, maturation

Another factor with a strong effect on lipid content and composition in animals is sex or sexual maturation (Nurnberg *et al.*, 1998); (De Smet *et al.*, 2004).

Kocour *et al.* (2007) reported that females of Hungarian synthetic mirror carp were fatter than males probably due to later maturation. In study with four common carp hybrids Buchtova *et al.* (2008) found only minor differences in lipid composition between males and females probably caused by different lipid content. Fajmonova *et al.*, (2003) did not find any sexual

dimorphism in lipid content and fatty acid composition in three-year-old carps.

### 1.5.6 Body tissue

Fish fillet is highly heterogeneous and is composed from several different tissues (e.g.: white muscle, red muscle, adipose tissue and skin). The tissues differ greatly in lipid content and therefore the lipids are not equally distributed in the fillet.

Variation in lipid content has an effect on fatty acid composition, independent of species or breed and dietary factors. In livestock, the content of saturated fatty acids (SFA) and mono unsaturated fatty acids (MUFA) increases faster with increasing fatness than does the content of PUFA (De Smet *et al.*, 2004). This was shown for bulls fed different diets (Raes *et al.*, 2003). Similar findings were reported for pork (Riley *et al.*, 2000) and sheep (Nurnberg *et al.*, 1998). The effect of fatness on the fatty acid composition can be explained to a large extent by differences in the fatty acid composition of the major lipid fractions and the relative contribution of these fractions to total lipids. Phospholipids are particularly rich in PUFA, whereas triacylglycerols contain much lower amounts of PUFA (De Smet *et al.*, 2004).

Decreasing level of PUFA with increasing fatness was also reported in several fish species: mapará (*Hypopthalmus sp.*) (Inhamuns & Franco, 2001), rainbow trout (*Oncorhynchus mykiss*) (Kiessling *et al.*, 2001). However, with MUFA being the main fatty acid class positively correlated with the level of fatness (reviewed by Henderson & Tocher (1987)).

### 1.5.7 Starvation

Purging of fish before slaughtering or delivery to market is a common practise in aquaculture to remove possible off-flavours and eliminate undigested food from intestine (Lim & Webster, 2006). It is usually done by moving the fish to clean water and starving them from few days to many weeks. The purging can also improve nutritional quality of the farmed fish by reducing excessive fat and increasing of n-3 HUFA percentage (Einen *et al.*, 1998); (Palmeri *et al.*, 2008). Einen *et al.* (1998) studied effect of starvation prior to slaughter in Atlantic salmon. They found significant but rather marginal effects of starvation on fatty acid composition in muscle, belly flap and liver. However, the fish used in the study had quite high muscle lipid content (16%) and therefore much bigger effects could probably be seen in fish with lower muscle lipid content.

Csengeri (1996b) studied effect of starvation on lipid content and composition in common carp. He observed that there was a consistent decrease in the oleic acid levels both in muscle and liver and that PUFA were somehow protected. He also reported that the effect of starvation was dependent on the previous feeding. Vacha *et al.*, (2007) studied effect of long term starvation on fatty acid composition in common carp fed either on cereals or natural feed only. The carps supplemented by cereals had high lipid content (> 10%) compared to the carps fed on natural feed only (1.8%). The biggest differences in fatty acid composition could be seen in the lean fish fed on natural feed only, where mainly decreased levels of PUFA were observed.

### 1.5.8 Processing and cooking

The last but not the least factor influencing lipid content and composition is processing and cooking. It was proved that especially the quality of fats and oils added during processing has a very strong influence on lipid composition (Ansorena & Astiasaran, 2004); (Sampels *et al.*, 2009). Sampels *et al.*, (2009) found very high variation of n-3/n-6 ratio in fish products being up to 400 lower than in the raw fish. They concluded that fat sources used during the processing and preparation have the largest impact on the food FA content and composition and proposed that it should be declared on the product label. The product lipid composition might further change when it is fried by the consumers (Ramirez *et al.*, 2005).

# 2 Objectives

The overall aim of this work was to evaluate factors influencing lipid content and composition in common carp muscle. This work is a part of a bigger project focused on improvement of carp muscle lipid quality in order to be used as a local healthy product for prevention and treatment of cardiovascular diseases. The goal is to develop a technology of carp culture with using long term sustainable alternative feedstuffs.

Specific objectives were to:

Investigate lipid content, lipid classes and fatty acid composition in three parts of common carp fillets (Paper I).

Examine the effect of genetic origin on lipid content and fatty acid composition in common carp (Paper I).

Study the effects of sesamin on fatty acid composition in common carp (Paper II).

Study the effect of sesamin on global gene expression *in vivo* in common carp (Paper II).

# 3 Material and Methods

The chapter shortly describes the material and methods used in the studies included in the thesis. For a more detailed description of each method see Papers I-II. An overview of the material and methods used is shown in Table 1.

## 3.1 Study design

In Paper I, samples from four different crosses of common carp (*Cyprinus carpio*) reared in an earthen pond were sampled (Mean weight 2 kg). They were reared on the basis of natural food (plankton and benthos) with cereal supplementation (wheat). The sampled crosses were a pure line of Hungarian mirror carp (M2 x M2), a hybrid line of two Hungarian mirror carps (M2 x L15), a hybrid line of Hungarian mirror carp and Israeli mirror carp (M2 x Dor70), and a hybrid line of Hungarian mirror carp and Northern mirror carp (M2 x M72). Lipid content and total lipid fatty acid composition (in total lipids, phospholipids and triacylglycerols) of fillets of four fishes per cross were analyzed. The samples consisted of one slice dissected from the dorsal fin to the ventral line, including 50% of the red muscle tissue.

Other Six randomly selected individuals of the M2xM2 strain (mean weight 2 kg) were used to analyze differences among fillet parts. Three tissue samples, white dorsal muscle (WM), red muscle (RM), and abdominal wall with adipose tissue (AW), were dissected from each fillet of the six individuals (Fig. 3). The samples were analyzed for lipid content, lipid class composition and fatty acid composition (in total lipid, phospholipids and triacylglycerols).

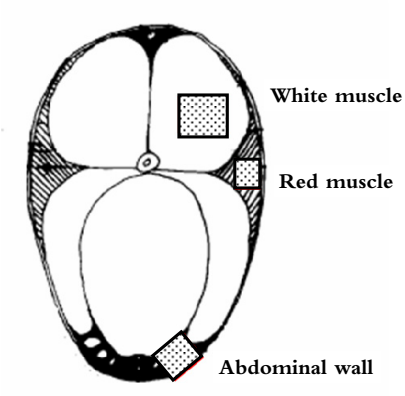


*Figure 3*. Sampling scheme (adapted from Kiessling *et al*. (1991))

Samples of plankton were collected from the pond in July, August and September 2007 and were analyzed for lipid content and fatty acid composition. In addition, samples of benthos and plankton were collected (3 occasions: May, July, and September; each in triplicate) and analyzed from three ponds in 2009 (stocked with common carp of similar fish weight and stocking density).

Table 1. Overview of study design for Papers I-II

| Study | Ia | Ib | Ic | II |
|---|---|---|---|---|
| Species | Common carp | Common carp | Plankton | Common carp |
| Size | 2 kg | 2 kg | | 1.7 kg |
| Type of culture | Ponds | Ponds | Ponds | Cages with recirculation system |
| Feeding | Natural food + cereals | Natural food + cereals | | Pelleted feed +/- sesamin |
| Tissues | White muscle | White muscle | | White muscle |
| | Red muscle | | | Hepatopancreas |
| | Abdominal wall | | | |
| Analyses | Lipid content | Lipid content | Lipid content | Lipid content |
| | Fatty acids | Fatty acids | Fatty acids | Fatty acids |
| | Lipid classes | | | Total cytochrome P450 |
| | | | | EROD |
| | | | | Global gene expression profiling |

In Paper II, two-year-old common carp (*Cyprinus carpio*) individuals (mean weight 830 g) were reared in six 1 $m^3$ tanks (6 fish per tank) connected to a recirculation system. The fish were fed diets with or without sesamin addition (0.58 g/100 g feed) for 9 weeks. Survival, specific growth rate and feed conversion ratio were calculated for each treatment. Samples of white dorsal muscle and hepatopancreas were taken from all fish. The white muscle samples were analysed for lipid content and fatty acid composition (in total lipids, phospholipids and triacylglycerols). The samples from hepatopancreas were analyzed for total content of cytochrome P450, EROD activity and global gene expression profiling.

## 3.2 Lipid analyses

### 3.2.1 Lipid extraction and fatty acid composition analyses

Lipids from tissues, diets and plankton were extracted by hexane–isopropanol method (Hara & Radin, 1978). Total lipids were fractionated by thin layer chromatography for separation of lipid classes (Pickova *et al.*, 1997). Fatty acids were methylated (Appelqvist, 1968) and analyzed with a Varian CP3800 gas chromatograph (Stockholm, Sweden) equipped with flame ionization detector, split injector and fitted with a 50 m length x 0.22 mm i.d. x 0.25 μm film thickness BPX 70 fused-silica capillary column (SGE, Austin, TX, USA) (Fredriksson Eriksson & Pickova, 2007). Fatty acids were identified by comparison with the standard mixture GLC-461 (Nu-check Prep, Elysian, MN, USA) using retention time. Peak areas were integrated by means of Star chromatography workstation software version 5.5 (Varian AB, Stockholm, Sweden). Fatty acids were quantified by use of the internal standard 15-methylheptadecanoate (Larodan Fine Chemicals AB, Malmo, Sweden).

### 3.2.2 Lipid classes composition

Analyses of lipid classes composition was performed according to (Olsen & Henderson, 1989) with minor modifications. Extracted lipid samples were applied by a Camag ATS 4 automatic TLC sampler on the pre-developed and activated TLC plates. The lipid classes were separated with hexane-diethyl ether-acetic acid (85:15:1, v/v) and detected by spraying with copper acetate-phosphoric acid solution. Quantitative analyses of the separated lipid classes were performed densitometrically by use of the Camag TLC scanner 3. The lipid classes were identified by comparing with an external standard (TLC 18-4A; Nu-Check Prep, Elysian, Minnesota, USA).

## 3.3 Sesamin analyses

Sesamin was analyzed from extracted lipid samples with HPLC according to Moazzami & Kamal-Eldin (2006). Separation was done on a silica column using hexane/1,4-dioxane (94:4, v/v) as mobile phase. Detection was done by a fluorescence detector (excitation wavelength 296 nm and emission wavelength 324 nm). External standards were used for identification and quantification.

## 3.4 Total content of cytochrom P450 and ethoxyresorufin O-deethylation

Microsomal fraction was prepared from the hepatopancreatic homogenate by means of Ca-aggregation method as described by Zamaratskaia *et al.* (2009). The total cytochrome P450 content was determined using the spectrophotometric method of Omura & Sato (1964), measuring the differences in the spectra (dithionite+carbon monoxide) – dithionite. The activities of 7-ethoxyresorufin O-deethylase were estimated using an HPLC-based method according to Zamaratskaia & Zlabek (2009).

## 3.5 Global gene expression profiling

The global gene expression analysis was performed using c DNA common carp microarray with 26K gene probes (carp ARRAY ver 5. (Williams *et al.*, 2008)). Total RNA was extracted from hepatopancreas using the TRIzol Plus RNA Purification Kit (Invitrogen 12183-555, Paisley, UK). Total RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was reversely transcribed to cDNA and labeled using the SuperScript Plus Indirect cDNA Labeling System (Invitrogen L1014-04, 05 and -06). The labeled cDNA was hybridized on the microarray using the Maui hybridization system (BioMicro Systems, Salt Lake City, UT, USA). The microarrays were scanned using the Agilent DNA microarray scanner and the obtained data were processed by BLUEFUSE software (BlueGnome, Great Shelford, Cambridge, UK).

## 3.6 Statistical analyses

The data were processed using data analysis software STATISTICA CZ, v. 8, StatSoft, Inc. All the values were expressed as mean ± standard deviation. For statistical analyses one tail Student t-test and one way ANOVA with

following Tukey's post-hoc test with statistical level of significance $\alpha = 0.05$ were used.

The microarray data were first normalized (Huber *et al.*, 2002) and corrected (Cleveland & Devlin, 1988). The differentially expressed genes were extracted using Q-values (Storey, 2002) and the control false discovery rate at the level of 10% (Benjamini & Hochberg, 2000). The up and down regulated genes were associated with the gene ontology terms to see which metabolic pathways were influenced by the sesamin treatment.

# 4 Summary of results

## 4.1 Paper I

The lipid content and composition varied considerably among the samples from different parts of carp fillet. The lowest lipid content was found in dorsal white muscle (0.95±0.14%), medium content in red muscle (16.7±5.0%), whereas the highest content was found in abdominal wall (30.2±7.8%). Lipid class composition of these different fillet parts showed that samples with higher lipid content had increased proportion of triacylglycerols. Consequently, abdominal wall with the highest lipid content was dominated by triacylglycerols whereas the white muscle had the highest contribution of phospholipids. The total lipid fatty acid composition differed greatly depending on the lipid content and ratio between phospholipids and triacylglycerols. The fatty acid composition of the leanest part, the white muscle contained a large proportion of n-3 highly unsaturated fatty acids and a ratio n-3/n-6 = 1.1, having a high proportion of phospholipids. The abdominal wall was rich in monounsaturated FA and had a lower ratio n-3/n-6 = 0.5.

The lipid content in white muscle of the cross M2 x M2 (1.40%) was slightly lower than the three other crosses (M2 x L15 = 1.80, M2 x M72 = 1.65, M2 x Dor70 = 1.85), although not significantly different. The total lipid fatty acid composition of the different crosses varied. In the M2 x M2 group, the proportion of n-3 PUFA was higher than in the other groups. However, there were no substantial differences when the fatty acid profiles of phospholipids and triacylglycerols were compared separately among the crosses. There was an inverse correlation between the lipid content and n-3 PUFA, indicating that the differences seen in the total lipid fatty acid

composition are most likely to be caused by the differences in lipid content and thus by a different proportion of phospholipids and triacylglycerols.

The zooplankton and benthos, which is the natural feed for carp, was sampled and analyzed. The zooplankton sampled from the pond in 2007 had a very favorable composition with high proportion of n-3 PUFA, especially EPA and DHA and a high ratio n-3/n-6 (2.4-7.5). There was an increasing trend in n-3 PUFA content, especially EPA and DHA, and n-3/n-6 ratio towards autumn. Similar trends can also be seen in plankton samples from 2009 (Fig. 5; unpublished data).

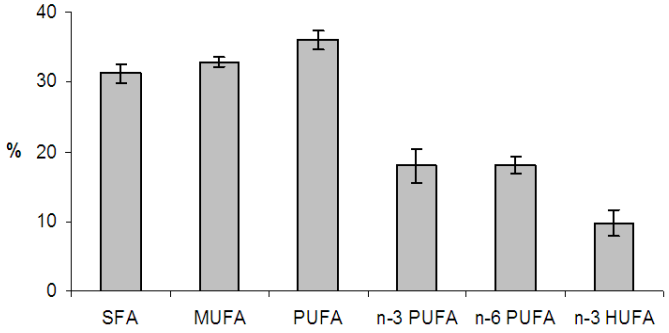


*Figure 4.* Fatty acid composition of benthos available in ponds in 2009. (sampled on three occasions; each occasion in triplicate; data are pooled, since there were no differences among the occasions)

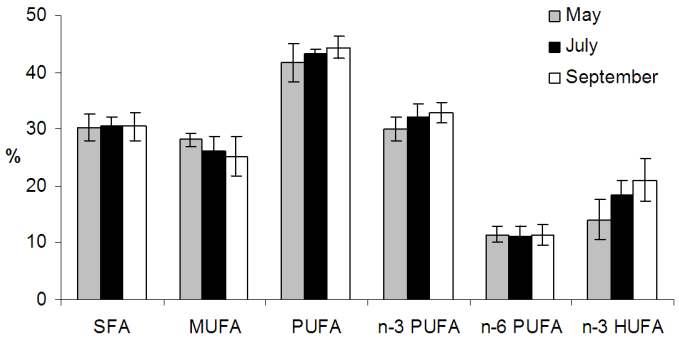


*Figure 5.* Fatty acid composition of plankton available in ponds in 2009 (sampled on three occasions; each occasion in triplicate)

The benthos sampled from the ponds in 2009 consisted mainly of *Chironomus plumosus* larvae. They were also rich in EPA and DHA and had

still a favorable n-3/n-6 ratio around 1, however, the level was much lower than in plankton (Fig. 4; unpublished data).

## 4.2 Paper II

There were no significant differences found in fish survival, specific growth rate, food conversion ratio and lipid content in white muscle between the fish supplemented with sesamin and control fish. Sesamin supplementation did not positively alter fatty acid composition in carp white dorsal muscle. Sesamin increased total cytochrome P450 content in hepatopancreatic microsomes as well as 7-ethoxyresorufin O-deethylase activity. Transcriptomic analysis, using microarray with 26K gene probes, found that expression of 662 genes was altered by sesamin in carp liver. However, it failed to establish any significant pattern of transcriptional response, including lipid biosynthetic genes. In conclusion, sesamin in our experiment did not increase n-3 HUFA biosynthesis in the common carp muscle.

# 5 General discussion

## 5.1 Different parts of fillet

### 5.1.1 Lipid content

A wide range of different fillet parts from different species has been used in studies focused on lipids and other quality parameters in fish which leads to varying data (Katikou *et al.*, 2001). However, lipids are not equally distributed in the fish fillet (Ackman, 1989). Consequently, lipid content, lipid class and fatty acid composition vary depending on where the sample is taken from (Aursand *et al.*, 1994). This was proven in salmon and our findings in Paper I show that common carp is not an exception.

In common carp, there are three main lipid deposition sides in fillet which are located in abdominal wall, dorsal adipose tissue and under skin. There are also two major muscle types in fish fillet, red and white muscle. White muscle forms the major part of the fillet. Red muscle is a thin longitudinal band located near the horizontal septa and skin. The two muscle types use different metabolic pathways to generate ATP and are active in different conditions. Red muscle uses efficient oxidative phosphorylation pathway to generate ATP and is used for normal slow swimming whereas white muscle uses glycolytic pathway and is used for fast rapid movement (Rome *et al.*, 1993; Johnston *et al.*, 1977).

(Johnston *et al.*, 1977) reported that common carp red muscle has lipid content ~ 20% and white muscle ~ 5%. Similar pattern was seen also in our study. We have seen quite similar values for red muscle (16.7%), however, we could see that lipid content in white muscle can vary considerably from ~ 1% (in dorsal part) to ~ 30% (in abdominal wall).

### 5.1.2 Lipid classes

The level of lipid content had high impact on lipid class composition in the samples from the three parts of the carp fillet (Paper I). It influenced mainly proportion between the two major lipid classes, phospholipids and triacylglycerols. Abdominal wall which had the highest lipid content had higher proportion of triacylglycerols and lower proportion of phospholipids whereas the opposite pattern was seen in the leanest part – white muscle.

It is known, that phospholipids serve as components of cell membranes and structures, while triacylglycerols serve as reserve of energy and they are mainly stored in adipose tissues (Sargent *et al.*, 1995). Thus, the higher level of fatness, the higher content of triacylglycerols can be observed.

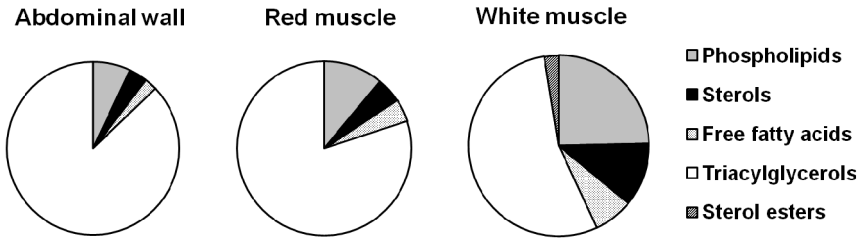


*Figure 6.* Lipid class composition (as relative %) of the three parts of carp fillet (from Paper I)

### 5.1.3 Fatty acids

Fatty acid composition of phospholipid and triacylglycerol fraction was quite similar in SFA (~ 30%) but very different in MUFA and PUFA. Phospholipid fraction had lower level of MUFA (~ 30%) and higher level of PUFA (~ 40%), especially EPA and DHA compared with triacylglycerol fraction (MUFA 60%; PUFA 10%). Consequently, the total lipid fatty acid composition was clearly affected by the lipid content in different samples. Thus the white muscle, being the leanest, was strongly influenced by phospholipids and had significantly higher proportion of n-3 HUFA, EPA and DHA.

Significant differences in FA composition were demonstrated between dorsal and ventral part in several other farmed species (Testi *et al.*, 2006). Similar findings were also reported by Kiessling *et al.* (2001) in studies with rainbow trout (*Oncorhynchus mykiss*). They found a strong interdependence between the relative proportion of total phospholipids and triacylglycerols with changes in lipid content. This was most prominent in white muscle. In

parallel with this change, in relative lipid class composition, a major effect was seen in FA composition within the total lipid.

### 5.1.4 The origin of fatty acids

The origin of the n-3 HUFA in the carp muscle is most likely the natural food, plankton and benthos, present in the ponds (Adamek *et al.*, 2004a; Prikryl, 1984). Our data shows (Paper I + unpublished data) that both plankton and benthos are rich source of n-3 PUFA and especially EPA and DHA having very favorable n-3/n-6 ratio. However, plankton had much higher levels of n-3 HUFA compared with benthos.

Seasonal variation in plankton FA composition was present in both years. There was an increasing trend in n-3 HUFA level towards the autumn period. This trend could be explained by changes in planktonic community. At the beginning of vegetation season, the zooplankton biomass was dominated by *cladocera*. Throughout the experiment there was a shift towards the smaller zooplankton species, *copepoda* and *rotifera*. High fish stock densities cause high predation pressure on zooplankton and suppress especially large species of *Daphnia* (Ingram, 2009). Consequently, the phytoplankton is not effectively controlled by small zooplankton species and efficiency and grazing of the high primary production is low as suggested by Potuzak *et al.* (2007). As a result of this effect, shift from *cladocera* towards *copepoda* could be seen in plankton community structure. The observed changes in plankton fatty acid composition throughout the experiment from EPA being the predominant n-3 HUFA towards the DHA are in agreement with Persson & Vrede (2006) who reported EPA to be typical of *cladocera*, whereas DHA is more frequent in *copepoda*.

In contrast to the low level of oleic acid in natural food, there was a high level of oleic acid in carp muscle. The origin is most likely from the supplemental cereal feeding. It is known that MUFA are produced by desaturation of saturated FA synthesized in the carp from this energy-rich feed (Csengeri, 1996a; Henderson, 1996). This could be probably improved by a change in feed composition by other vegetable sources than cereals.

### 5.1.5 Nutritional value of carp fillet

We concluded that common carp has a potential to become an attractive fish in terms of lipid content and composition. The nutritive value of the lipids in the present carp production is high, especially in the lean parts of the fillet, in relation to the amount of n-3 PUFA in the diet of people in central Europe. We propose that carp muscle has favorable FA composition and should be regarded as a healthy product. In addition, there could be several

possibilities to further improve the lipid composition of cultured carp in the traditional pond production.

The way to go in carp culture is probably not in using fish oil because of its general shortage and not long term sustainability, but supplemental feeds based on vegetable components containing ALA. Such components could be rapeseed and linseed. In our trials with newly developed rapeseed/linseed mixture pellets, we could see carp fillets containing 300 mg EPA+DHA and 1 g of n-3 PUFA per 200 g serving (unpublished results). These results are promising since they are quite close to values generally recommended for prevention of cardiovascular diseases.

Further improvement might be reached when we understand more the mechanisms and regulation of n-3 HUFA biosynthesis. Since there are studies showing that n-3 HUFA content in fish muscle is highly heritable trait (Leaver *et al.*, 2011), we could probably further improve carp quality by identifying and selecting lines with high n-3 HUFA content.

## 5.2 Effect of genetic origin

The four carp crossbreeds analyzed in our study (Paper I) had quite similar FA compositions in the white dorsal muscle. The only hybrid which was different, when looking at the total lipid FA composition, was the pure line of Hungarian mirror carp (M2 x M2). Nevertheless, there was no difference when phospholipid and triacylglycerol fractions were analyzed separately. So the differences seen in the total lipid FA composition could probably be ascribed to differences in the lipid content and consequently to different proportion between the two lipid fractions. The limitation of this study is in genetical closeness of the four studied crossbreeds. It would be interesting to conduct a similar experiment with carp lines which are genetically very distant, e.g.: the two subspecies of common carp *C.c. carpio* and *C.c. haematopterus*.

## 5.3 Effect of sesamin

The aim of the study in Paper II was to investigate if diet with sesamin has a positive effect on fatty acid composition in common carp muscle as was seen in salmonids (Trattner *et al.*, 2008a; Trattner *et al.*, 2008b). Unfortunately, dietary sesamin did not alter positively fatty acid composition of carp muscle in the present study.

In Paper II, we discussed several explanations for the discrepancy.

1) An evolutionary aspect should be considered. Salmonids as predators are not naturally consuming this type of vegetable substances whereas cyprinids being the omnivores usually acquire such compounds in their food. It was shown also in mammals that there are species-dependent differences in the physiological response to dietary lignans (Kushiro *et al.*, 2004).

2) Suppression of HUFA biosynthesis by n-3 HUFA content in experimental diets was another possible explanation.

3) We used in our experiment pure sesamin instead of equimixture of sesamin/episesamin which was used in the study by Trattner *et al.* (2008a). Therefore, episesamin rather than sesamin might be responsible for the observed effects in salmonids.

4) The aspects of different rates of HUFA biosynthesis related to age and size also need to be investigated since it might result in various effects of sesamin.

We could see in our experiment that carps supplemented with sesamin had higher EROD activity and higher content of total CYP P450, as reported in rainbow trout liver (Trattner *et al.* 2008a). This indicates that carp also recognizes sesamin as a xenobiotic compound.

The transcriptomic profiling with cDNA microarray showed that sesamin supplementation altered expression of 662 genes in carp liver. However, we were not able to define any viable pattern to the responding genes that might signal changes in intermediary and particularly lipid metabolism.

In conclusion, we have not seen in our experiment with carp any positive alteration of HUFA biosynthesis. So probably it would be interesting to investigate more deeply mechanism of sesamin action and metabolism in salmonids, where the sesamin had positive effects, before trying to study it in other fish species. In the future, it may happen that also other bioactive compounds which influence positively n-3 HUFA biosynthesis will be identified and used in fish culture.

# 6 Conclusions

The FA composition is different in PL and TAG fraction. PL have a higher level of n-3 HUFA in comparison with TAG. FA composition in total lipid of different parts of the fillet depends strongly on the lipid content, because of the influence on the ratio of the two main fractions - PL and TAG.

We concluded that common carp has a potential to become an attractive fish in terms of lipid content and composition. The nutritional value of the lipids in carp fillet is high, especially in the leaner parts of the fillet, considering the amount of n-3 PUFA in the diet of the Central European population.

The four carp crossbreeds analyzed in our study had quite similar FA compositions in the white dorsal muscle. Differences seen in the total lipid FA composition were ascribed to differences in the lipid content and consequently to different proportion between PL and TAG.

Sesamin supplementation had no impact on fish production performance such as weight, specific growth rate, food conversion ratio and lipid content. Addition of sesamin did not alter fatty acid composition of carp muscle in the present study. Sesamin increased total cytochrome P450 content in hepatopancreatic microsomes as well as EROD activity. The transcriptomic profiling with cDNA microarray showed that sesamin supplementation altered expression of 662 genes in carp liver. However, we were not able to define any viable pattern to the responding genes that might signal changes in lipid metabolism. Together these results indicate that sesamin is ineffective in the common carp as a means of achieving an altered tissue lipid composition.

Since carps have all the required enzymes for the n-3 HUFA synthesis, it is tempting to explore how this synthesis is regulated and if it is possible to increase the effectiveness of this bioconversion.

# 7 Future research

This thesis evaluated several factors influencing lipid content and composition in common carp muscle. Some specific areas of future interest are:

Influence of supplemental diets based on vegetable feedstuffs on the lipid composition of common carp cultured in ponds.

The effect of starvation period on carp lipid metabolism and composition.

Differences in lipid metabolism between two common carp subspecies, *C. c. carpio* and *C. c. haematopterus*.

The use of finishing feeding strategy in common carp.

Screening for bioactive substances which could be modulators of lipid metabolism.

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