UNIVERSITY OF SOUTH BOHEMIA IN ČESKÉ BUDĚJOVICE

FACULTY OF FISHERIES AND PROTECTION OF WATERS

Improvement of fatty acid composition in common carp (*Cyprinus carpio*)

Vylepšení kompozice mastných kyselin u kapra obecného (*Cyprinus carpio*)

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Czech Republic, Vodňany, 2011

I thereby declare that I wrote the Ph.D. thesis myself using results of my own work or collaborative work of me and colleagues and with help of other publication resources which are properly cited.

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Chapter 1

General 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 2987400 tons and 144750 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., 2004). 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 decades. It will not be possible to further increase the fish capture in world-wide scale without risk of overfishing and 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 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. As the aquaculture is expanding, 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 et al., 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. It is an example of long term sustainable production without relying on the supply of fish oil and fish meal which is the problem in production of predatory species. There are two sources of n-3 HUFA in carp produced in ponds. 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 able to bio-convert ALA to

EPA and DHA (Farkas, 1984; Olsen et al., 1990; Tocher, 2003; Zheng et al., 2004). 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 et al., 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 usually 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).

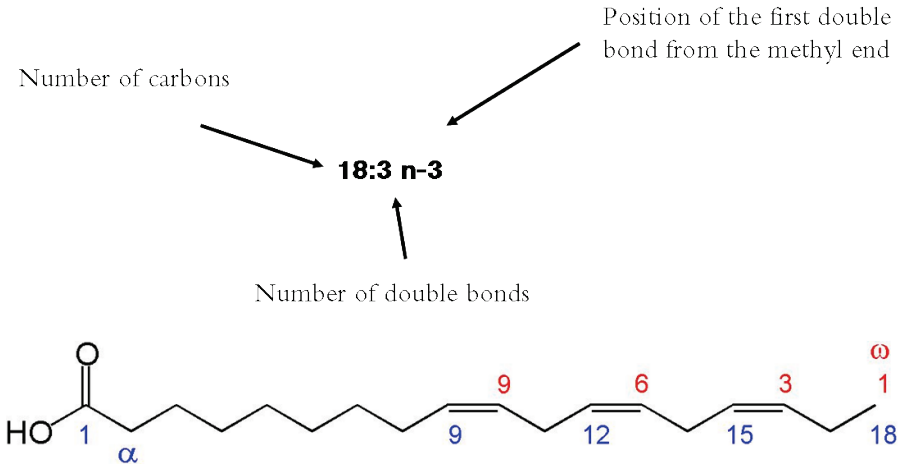

Figure 1. Principle of fatty acid nomenclature.

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 (SFA). The saturated fatty acids can be further modified by inserting a double bond to form mono unsaturated fatty acids (MUFA) 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 HUFA by desaturases and elongases (Fig. 2) (Voss et al., 1991).

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)). Regulation of HUFA biosynthetic pathway is described in details in chapter 1.5.

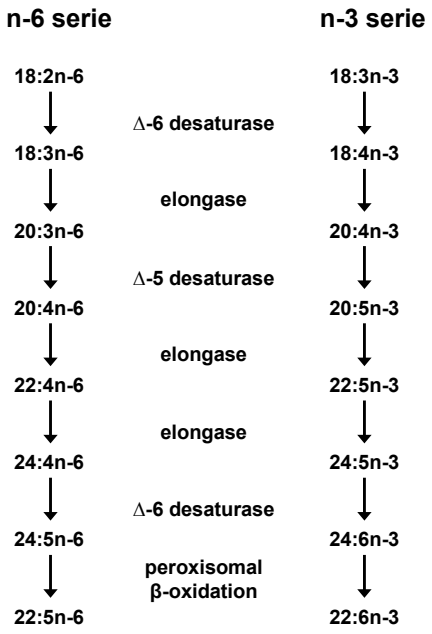

Figure 2. Pathways of HUFA biosynthesis from C18 fatty acids, adapted from Voss et al. (1991).

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 et al., 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 et al., 2009).

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 et al., 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 1:1 whereas in the western diet it exceeds 1:15 (Leaf et al., 1987; Simopoulos, 2008). This dietary change is associated with many life style 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 et al. (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, agencies, 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. HUFA biosynthesis

1.5.1. HUFA biosynthesis in common carp

The HUFA biosynthetic pathway in fish was established using rainbow trout hepatocytes (Buzzi et al., 1996). It is anticipated and also suggested by accumulated evidence that the same pathway exist also in other fish species including common carp (Tocher, 2003). Common carp has been shown to be able to elongate and desaturate n-3 and n-6 HUFA from its C18 precursors. It was shown by Farkas et al. (1978) using injection of radiolabled sodium acetate into the common carp juveniles with followed detection of incorporated radioactivity into different fatty acids. In additional experiments Farkas (1984) used slices from common carp livers incubated with radiolabled fatty acids. Tocher et al. (1999) showed common carp ability to convert ALA to HUFA in cell lines. It was also shown by feeding studies that essential fatty acid requirements of common carp can be satisfied by 18C PUFA which suggests that common carp is able to convert them to HUFA (RadunzNeto et al., 1996; Takeuchi, 1996). Despite the large number of studies investigating common carp ability to produce HUFA from C18 precursors, the molecular mechanisms behind the HUFA

biosynthesis are not fully understood and the genes coding the responsible enzymes remain to be identified and characterised in common carp.

1.5.2. Genes related to HUFA biosynthesis

The first characterised fish desaturase was from model species zebrafish (*Danio rerio*). It was done by Hastings et al. (2001) who used heterologous expression in the yeast *Saccharomyces cerevisiae*. They found that zebrafish has a bifunctional Δ-5/Δ-6 enzyme which posses the capacity to desaturate both types of substrate. In contrast, other characterised fish desaturases are either unifunctional or have major activity towards the one substrate with only residual capacity to desaturate the other one (Hastings et al., 2004; Monroig et al., 2010b; Zheng et al., 2004; Zheng et al., 2005a). In Atlantic salmon three different desaturases with Δ-6 activity were identified and characterised (Monroig et al., 2010b). The only HUFA related gene characterised for common carp is Δ-6 desaturase (GenBank accession no. AF309557) (Zheng et al., 2004). It included open reading frame of 1335 base pairs specifuing protein of 444 amino acids. It was more specific towards the n-3 substrate and it showed low Δ-5 desaturase activity. Thus the separate Δ-5 desaturase probably remains to be identified for common carp.

Table 1. Overview of characterised HUFA biosynthetic genes in Atlantic salmon (*Salmo salar*).

Gene name	GenBank accession No.	Product	Characterised by
D6fad_a	AY458652	delta-6 fatty acyl desaturase D6fad_a	(Zheng et al., 2005a)
D6fad_b	GU207400	delta-6 fatty acyl desaturase	(Monroig et al., 2010b)
D6fad_c	GU207401	delta-6 fatty acyl desaturase	(Monroig et al., 2010b)
Fadsd5	AF478472	delta-5 fatty acyl desaturase	(Hastings et al., 2004)
elov12	FJ237532	polyunsaturated fatty acid elongase elov12	(Morais et al., 2009)
elvol5a	AY170327	polyunsaturated fatty acid elongase elvol5a	(Hastings et al., 2004)
elovl5b	FJ237531	polyunsaturated fatty acid elongase elovl5b	(Morais et al., 2009)
elovl4	HM208347	elongation of very long chain fatty acids-like 4	(Carmona-Antoñanzas et al., 2011)

There are several elongases identified in mammals with three involved in elongation of PUFA (ELOVL2, ELOVL4 and ELOVL5) (Jakobsson et al., 2006). ELOVL2 predominantly elongate C20 and C22 whereas ELOVL5 primarily elongate C18 and C20 (Leonard et al., 2000). Agaba et al. (2004) characterised a multifunctional elongase from zebrafish with ability to elongate C18, C20, C22 as well as MUFA and PUFA. Hastings et al. (2004) characterised ELOVL5 in Atlantic salmon with activity to elongate mainly C18 and C20. Morais et al. (2009) Characterised ELOVL2 and second ELOVL5b from Atlantic salmon and showed that the elongase ELOVL5b primarily elongate C18 and C20 and ELOVL2 elongase predominantly elongate C20 and C22. ELOVL4 has been characterised in zebrafish (Monroig

et al., 2010a) and in Atlantic salmon (Carmona-Antoñanzas et al., 2011) with ability tu convert C20 and C22 up to C36. However, no elongase have so far been identified and characterised for common carp.

1.5.3. Regulation of HUFA biosynthesis

Nutritional factors such as content of fatty acids (Jump et al., 1999) and bioactive compounds in the diet affect the expression of genes involved in lipid metabolism (Trattner et al., 2007; Trattner et al., 2008a; Trattner et al., 2008b). Generally, both desaturases and elongases are up-regulated in fish fed diet with 18C precursors (vegetable oils) compared with fish fed diet containing HUFA (fish oil) (Leaver et al., 2008; Morais et al., 2009; Tocher et al., 2001; Zheng et al., 2005b). Environmental factors have also been shown to influence the expression of Δ6 desaturase in fish (Zheng et al., 2005b). For more detailed information see the review by Vagner et al. (2011).

Hepatic Δ-6 and Δ-5 desaturases are coordinately regulated. The rate of transcription of Δ-6 desaturase is positively correlated with the nuclear concentration of SREBP-1 and stimulated by PPARα activators. The nuclear content of SREBP-1 is increased by insulin and glucose, and back regulated by (n-6) and (n-3) PUFA. Different PPARα activators e.g. fibrates induce Δ-6 desaturase enzymatic activity by an increased rate of Δ-6 desaturase gene transcription caused by the enhanced binding of activated-PPARα to the Δ-6 desaturase promoter (He et al., 2002). Elongases are regulated by the nuclear receptor liver X receptor α (LXR) and the transcription factor SREBP-1c (Qin et al., 2009), which both in turn are influenced by the concentration of PUFAs. PUFAs repress expression of SREBP-1c and ELOVL5, but when combined with LXR ligand stimulation, which increases SREBP-1c mRNA and nuclear SREBP-1c, ELOVL5b mRNA levels are restored to normal. ELOVL5 is also target for several miRNAs in zebrafish (zebrafish miRNA database, http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer4.pl).

Dreesen et al. (2006) found in human genome protein non coding antisense RNA gene that functions as a naturally occurring ‘‘cis-antisense’’ regulator of Δ-5 desaturase gene expression. Anti-sense transcripts may regulate gene expression by interfering with gene transcription and mRNA processing, accelerating mRNA decay, and slowing translation of mRNA into protein. Dreesen et al. (2006) provided evidence that coexpression of Reverse Δ-5 desaturase non-coding RNA (RevΔ5ase ncRNA) decreased the Δ-5 desaturase enzymatic activity > 70%. RevΔ5ase ncRNA likely exerts its influence through the binding of complementary regions of the Δ-5 desaturase transcript. The resulting double stranded RNA complex could accelerate Δ-5 desaturase mRNA decay, and/or interfere with Δ-5 desaturase mRNA translation. However, it is not known if the RevΔ5ase ncRNA exist in fish.

Most likely miRNAs and other small RNAs play a similar critical role in more or less all stages of uptake, transport, elongation, desaturation, β-oxidation and composition of n-3 and n-6 lipids in freshwater fish. However, until now no reports of miRNA regulation of HUFA biosynthesis have been made for carp to our knowledge.

1.6. Factors influencing lipid content and composition

1.6.1. Species

Species of the animal is the largest 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 (Fontagné-Dicharry et al., 2010). Some fish species have very low lipid content in fillet (less than 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 et al., 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 et al., 1987).

1.6.2. Nutrition

"You are what you eat" – 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., 2004; Adámek 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, 1996).

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 et al., 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.

1.6.3. 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. (2008b) 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 hepatocytes showed that sesamin/episesamin exposure led to increased elongation and desaturation of 14C ALA to DHA indicating that sesamin has modulatory effects on lipid metabolism leading to increased levels of DHA and higher β oxidation activity (Trattner et al., 2008a). 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).

1.6.4. 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 (De Smet et al., 2004; Karamichou et al., 2006; Khang et al., 2007).

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 ($h2 = 0.77 \pm 0.14$). 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 not many 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.

1.6.5. Sex, maturation

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

Kocour et al. (2007) reported that females of Hungarian synthetic mirror carp were fattier 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.

1.6.6. 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 SFA and 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 et al., 2001), rainbow trout (Kiessling et al., 2001). However, with MUFA being the main fatty acid class positively correlated with the level of fatness (reviewed by Henderson et al. (1987)).

1.6.7. 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 et al., 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 (1996) 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.

1.6.8. 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 et al., 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).

1.7. The aim of this thesis was:

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

Study the effects of bioactive compound sesamin on common carp fatty acid composition and global gene expression *in vivo* (Paper II).

Examine whether the lipid composition of common carp muscle can be improved by different rearing strategy in ponds and become an important source of n-3 highly unsaturated fatty acids in human nutrition (Paper III).

Screen and *in vitro* evaluate the effects of other bioactive compounds with potential to influence lipid composition (Paper IV).

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Chapter 2

Differences between lipid content and composition of different parts of fillets from crossbred farmed carp (*Cyprinus carpio*)

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Differences between lipid content and composition of different parts of fillets from crossbred farmed carp (*Cyprinus carpio*)

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Abstract Common carp (*Cyprinus carpio*) is, globally, one of the most important farmed fish species. We have analyzed carp from a natural pond-production system in South Bohemia. Ongrowing fish had access to an abundant plankton population which was supplemented with cereals. Fatty acid composition (FA) was investigated in three parts of fillets from four crosses of carp. The FA composition of the leanest part, the dorsal white muscle was similar to that of marine-farmed species; it contained a large proportion of n-3 highly unsaturated FA, the n-3/n-6 ratio was 1.1, and the proportion of phospholipids was high. The abdominal wall is rich in monounsaturated FA, the n-3/n-6 ratio is lower, 0.5, and it is more affected by the cereal feed. We concluded that the lipid composition of all these carp tissues can be improved in terms of healthy FA profile.

Keywords Fatty acids · Freshwater fish · n-3/n-6 Ratio · PUFA

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Introduction

Common carp (*Cyprinus carpio*) is, globally, one of the most important farmed species—FAO statistics report world production of over 3 million tons in 2005.

Decreasing fisheries and fish availability increase the price of fish and fish products for both direct consumption and for fish feed raw materials. Lipid dietary requirements of fish are important to ensure the optimum growth and welfare of the fish in culture and also to meet the consumers' expectations of a healthy product. Therefore, great attention is currently directed toward suitable mixtures of fish-feed lipids to meet the requirements of the fish. It is also important to maintain the major attribute in the western world of fish as a healthy food in terms of lipid composition and, especially, a high content of omega-3 highly unsaturated fatty acids (n-3 HUFA). Thus, the challenge is to develop diets which fulfill the lipid requirements of the fish and make the fish flesh a healthy product from a consumer's point of view.

Fish oil, the main source of oil in feed for salmonids and other predatory fish, contains the two most important HUFA, eicosapentaenoic acid, EPA, (20:5n-3), and docosahexaenoic acid, DHA, (22:6n-3), which make the flesh of the fish a healthy product. Mozaffarian and Rimm (2006) have, in a meta-study, demonstrated the advantage of intake of these fatty acids in relation to cardiovascular disorders. Beneficial

effects on neurodevelopment during gestation and infancy were also demonstrated. The major conclusion from this study was that the benefits of fish intake exceed the potential risks of possible harmful effect of pollutants (Mozaffarian and Rimm 2006). Vegetable oils have been extensively studied as replacements for fish oils. Vegetable oils often result in increased 18:2n-6 content and reduced n-3 HUFA, resulting in a lower n-3/n-6 ratio in the edible fish tissues (Bell et al. 2001). Rapeseed oil (RO) is regarded as one of the best substitutes for fish oil both for nutritional reasons and because acceptance by the fish is high (Geurden et al. 2005; Pettersson et al. 2008).

Because the common carp is an omnivorous species, it has been suggested it does not have the same n-3 HUFA requirements as cold-water salmonid fish (Henderson and Tocher 1987).

As both freshwater and saltwater fish live in similar conditions and are affected by the same factors, lipid content and composition of freshwater fish muscle will be affected similarly as in marine fish. Many factors in the production system, including the diet and its fatty acid composition, are of major importance. Minor compounds among dietary lipids often have a large biological effect on the lipid composition of fish (Pickova and Mørkøre 2007). Another important factor affecting lipid content is genetic origin, but no relevant data on this are available for carp (Fauconneau et al. 1995; Buchtová et al. 2007). These studies present total lipid profiles of the crosses which have been investigated and no differences among them was observed.

In several studies, environmental and nutritional effects on fatty acid composition in common carp have been investigated (reviewed in Steffens 1997). However, semi-extensive farming of carp in classical production systems in Europe is based on cereal feeding as an energy supplement to the plankton naturally produced in the pond systems. Cereals are rich in monounsaturated fatty acids and linoleic acid (18:2n-6) and plankton naturally produce high levels of n-3 HUFA, including EPA and DHA (Linko et al. 1992).

The objective of this study was to evaluate whether different lipid quality could be detected among four strains of common carp. Lipid class and the FA composition of total phospholipids and triacylglycerols were investigated for white muscle (WM), red muscle (RM), and adipose tissue from the abdominal wall (AW) of common carp farmed on the basis of natural food supplemented by cereal feeding (wheat).

Material and methods

Fish and sampling

Carp were sampled in September 2006 and 2007 from the farming facilities of the Research Institute of Fish Culture and Hydrobiology at Vodňany, Czech Republic. The analyses were carried out at the Department of Food Science, SLU, Uppsala, Sweden. The fish were cultured in one earthen pond in the Vodňany region (Czech Republic). They were reared on the basis of natural feed (plankton and benthos in the pond) and supplemented by cereal feeding (wheat). Mean weight of the fishes was ~2 kg and the age was 3+. Sampling was performed after the fish had been transferred to a tank and starved for two days at 10°C. The fish were killed with a blow to the head and filleted. One fillet, with skin, per fish was frozen (−40°C) for analyses. The frozen samples were transported to the laboratory.

In 2006 samples of four different crosses of carp were collected, a pure line of Hungarian mirror carp (M2 × M2), here called BP, a hybrid line of two Hungarian mirror carp (M2 × L15), BL, a hybrid line of Hungarian mirror carp and Israeli mirror carp (M2 × Dor70), PP, and a hybrid line of Hungarian mirror carp and Northern mirror carp (M2 × M72), PL. Fillets from four fish per genetic strain group were analyzed for lipid content and the fatty acid composition of total lipids, phospholipids, and triacylglycerols. The samples consisted of one slice dissected from the dorsal fin to the ventral line, including 50% of the red muscle tissue.

In September 2007 six randomly selected individuals of the BP (M2 × M2) strain were used for analyses. Three tissue samples, white dorsal muscle (WM), red muscle (RM), and adipose tissue with ventral muscle (AW), were taken from each fillet from six fish. Samples of plankton were collected in July, August, and September one week before slaughter of the fish and analyzed for lipid content and fatty acid composition of total lipids (Table 1).

Table 1 Main fatty acid composition of plankton, harvested in 2007, total lipids, as a percentage of total fatty acids, analyzed in duplicate from each sampling occasion

	July	August	September
Lipid content	1.0	1.4	0.8
Fatty acid			
14:0	3.89	3.88	4.06
16:0	19.1	18.7	15.5
16:1n-7	7.45	8.77	7.97
18:0	4.66	5.79	3.10
18:1n-9	14.4	11.4	7.35
18:1n-7	3.94	4.02	2.40
18:2n-6	6.74	6.09	3.58
18:3n-3	11.8	10.5	14.6
18:4n-3	2.82	1.89	7.10
20:4n-6	6.84	6.86	3.06
20:3n-3	0.35	0.44	0.51
20:4n-3	0.80	0.82	1.33
20:5n-3	12.3	13.1	16.6
22:5n-3	0.42	0.59	0.87
22:6n-3	4.52	7.19	11.3
ΣSFA	27.6	28.4	22.6
ΣMUFA	25.8	24.2	18.2
ΣPUFA	46.6	47.5	59.2
Σn-3 PUFA	33.0	34.5	52.3
Σn-6 PUFA	13.6	12.9	6.95
Σn-3/Σn-6	2.43	2.66	7.52

The abbreviations are explained in the caption to Fig. 1

Biochemical analysis

Lipid extraction

Five grams of sample were extracted. The samples were cut and homogenized with 50 ml HIP (hexane–isopropanol 3:2, *v/v*) by use of an Ultra-Turrax (Janke and Kunkel, IKA Werke, Germany; 3 × 30 s). Homogenate was transferred to a centrifuge bottle. The container was rinsed with another 25 ml HIP and this also was transferred to a centrifuge bottle. Na_2S0_4 solution (6.67%, 32.5 ml) was then added to remove non-lipids (Hara and Radin 1978). The samples were then shaken vigorously and centrifuged for 5 min at 4,000 rpm and 18°C. The lipid phase was transferred to a pre-weighed evaporation flask and 5 ml hexane was added to the non-lipid phase, which was shaken and centrifuged once again for 5 min at 4,000 rpm and 18°C. The hexane phase was transferred to the evaporation flask and the combined hexane extract was evaporated with a Büchi R-205 Rotavapor, equipped with a Büchi heating bath and a Vacuubrand MZ 2C vacuum pump. The evaporation bottle with the dry sample was weighed to determine the lipid content of the sample. The dry lipid samples were dissolved in 1 ml hexane and transferred to glass vials for further analysis. The white muscle lipid content was determined by weighing the lipid dissolved in 1 ml hexane on a micro balance (Mettler Toledo, UMT 2) for more exact measurement. In further analyses all samples were analyzed in duplicate.

Lipid class composition

White muscle, red muscle, and adipose tissue lipids were separated by thin-layer chromatography (TLC) to determine the composition of the different lipid classes. Precoated silica gel 60 TLC plates (20 cm × 10 cm; 0.20 mm layer; Merck, Darmstadt, Germany) were used as stationary phase. The analysis was performed according to Olsen and Henderson (1989) with minor modifications. The TLC plates were pre-developed to the full length by placing the plate in a hexane–diethyl ether–acetic acid (85:15:1, *v/v*) as mobile phase for 15 min. After drying, the top 1 cm of the silica layer was scraped off. The plate was then activated at 110°C for 1 h. Plates were placed in desiccator overnight. Samples were prepared in vials (100 µl) at a concentration of 1 µg/µl. Samples were applied to the TLC plates by use of a Camag ATS 4 automatic TLC sampler. The lipid classes were separated by placing the TLC plate in the chamber with hexane–diethyl ether–acetic acid (85:15:1, *v/v*) as mobile phase for 15 min. After drying, the plate was sprayed heavily with copper acetate–phosphoric acid solution (3 g $(CH_3COO)_2Cu$, 8 ml concentrated phosphoric acid, 92 ml ddH_2O). The plate was then heated at 160°C for 15 min then cooled to room temperature. The proportions of the different classes of lipid were measured densitometrically by use of the Camag TLC scanner 3.

Lipid classes were identified by comparing the samples with an external standard (TLC 18-4A; Nu-Check Prep, Elysian, Minnesota, USA). For data filtering, Savatisky-Golay 7 mode and manual baseline correlation were used.

Lipid class separation

Total lipids (TL) were fractionated on 20 × 20 cm TLC plates coated with silica gel 60 F_{254} (Merck) for separation of lipid classes in accordance to Pickova et al. (1997). Two milligram total lipids were applied in duplicate as a 2-cm band on the TLC plate together with a control sample and a standard. The lipids were separated by placing the plate in a hexane–diethyl ether–acetic acid (85:15:1, *v*/*v*) as mobile phase for 1 h. The control sample and the standards were revealed with iodine, with the other lipids covered with a glass plate. Total phospholipids, triacylglycerols, and free fatty acids were scraped from the plate and extracted in three steps. Total phospholipids were extracted sequentially with 3 ml chloroform–methanol (1:1, *v*/*v*), 2 ml chloroform–methanol (2:1, *v*/*v*), and 2 ml chloroform. Triacylglycerol fractions and free fatty acids were extracted three times with chloroform (3, 2, and 2 ml, in sequence). Lipid fractions were transferred to smaller tubes and solvents were evaporated under N_2 until dryness. All samples were then dissolved in 0.50 ml hexane.

Preparation of fatty acid methyl esters (FAME)

Preparation of FAME was performed in accordance with Appelqvist (1968). NaOH in dry methanol (0.01 M, 2 ml) was added to each tube. All samples were shaken vigorously and placed in a heating block at 60°C for 10 min. BF_3 (14% boron trifluoride–methanol complex, 3 ml) was added as esterification agent and the vials were reheated at 60°C for 10 min. All samples were cooled in ice–water and 2 ml 20% NaCl and 2 ml hexane were added. The tubes were shaken vigorously and left to stand for 20 min. The upper phase containing the FAME was transferred to a smaller tube and evaporated under nitrogen gas. The dry samples were dissolved in hexane, vortex mixed, and stored at −20°C until gas chromatographic analysis.

Gas chromatography

FAME were analyzed with a Varian (Stockholm, Sweden) CP3800 gas chromatograph equipped with flame ionization detector (FID) and split injector and fitted with a 50 m length × 0.22 mm i.d. × 0.25 μm film thickness BPX 70 fused-silica capillary column (SGE, Austin, TX, USA) (Fredriksson-Eriksson and Pickova 2007).

Samples (1 μl) were injected in split mode by use of a CP8400 autosampler (Varian). The split ratio was 1:10. Column temperature was programmed to start at 158°C for 5 min, then increase at 2°/min from 158 to 220°C and remain at 220°C for 8 min. Injector and detector temperatures were 230 and 250°C, respectively. Fatty acids were identified by comparison with the standard mixture GLC-68 A (Nu-check Prep) and use of retention times. Peak areas were integrated using a Star chromatography workstation with software version 5.5 (Varian). The carrier gas was helium (22 cm/s, flow rate 0.8 ml/min). Make up gas was nitrogen.

All solvents and chemicals were of analytical-grade and were purchased from Merck unless otherwise stated.

Statistics

Means and standard deviations were calculated for all analyses. The significance of fatty acids among tissues within lipid class was analyzed by ANOVA. Comparisons of fatty acids between tissue phospholipids, triacylglycerols, and total lipids were tested by use of Students' *t*-test.

Results

Analysis of plankton samples showed that the n-3 fatty acid composition increased slightly from summer to autumn, and the PUFA n-3/n-6 ratio changed accordingly. The lipid content was highest in August (Table 1).

The lipid content of the carp fillets from the 2006 season was analyzed ($n = 4$/group). The lipid content was BL = 1.80%, PL = 1.65%, PP = 1.85%, and the lipid content of the group BP was slightly lower 1.40%, although not significantly different (standard deviation (SD) = 0.45, 0.47, 0.29, 0.33, respectively). The amounts of the fatty acid groups (saturated, monounsaturated, n-3 and n-6 polyunsaturated) in the flesh of the different crosses varied. Fatty acid groups are shown in Fig. 1 ($n = 4$/group). In the BP group, the proportion of n-3 PUFA is higher than in the other groups ($P < 0.05$). Figure 2 shows a plot of lipid content and n-3 PUFA. Table 2

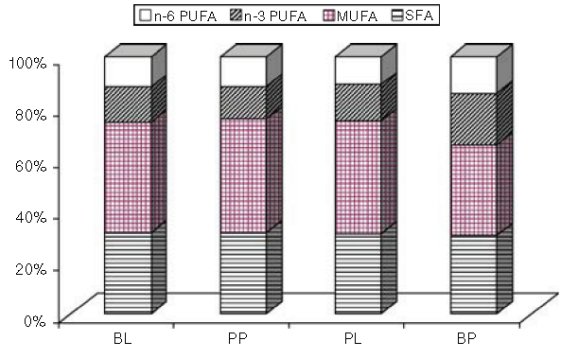

Fig. 1 Composition of fatty acid groups of total muscle lipids from four different carp crosses: BL = (M2 × L15); PP = (M2 × Dor70); PL = (M2 × M72); BP = (M2 × M2); PUFA = polyunsaturated fatty acids, MUFA = monounsaturated fatty acids, SFA = saturated fatty acids

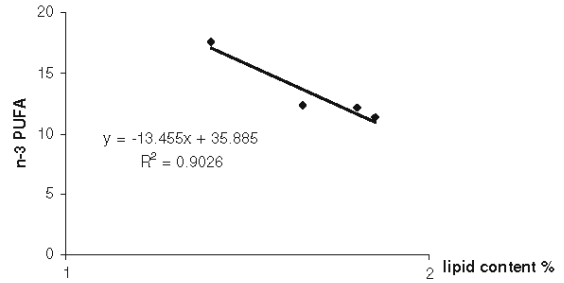

Fig. 2 Relationship between lipid content (%) and n-3 PUFA (as a percentage of total fatty acids of total lipids) from four different carp crosses

shows the profiles of individual fatty acids in the muscle phospholipids and triacylglycerols. No substantial differences among the fatty acid profiles were found.

In the fish from 2007, the lowest lipid content was found in the white muscle (0.95 ± 0.14%); it was medium in red muscle (16.7 ± 5.0) and highest in the abdominal wall (30.2 ± 7.8).

The lipid class composition of the different tissues is shown in Fig. 3. In samples with greater lipid content the proportion of triacylglycerols was higher. Consequently, abdominal wall with the highest lipid content was dominated by triacylglycerols. The contribution of total phospholipids was largest in white muscle, ~25% of the total lipid.

The fatty acid composition of total lipids (TL), total phospholipids, triacylglycerols, and free fatty acid (FFA) fractions of white muscle are shown in Table 3. The total phospholipid fraction contained a higher level of n-3 HUFA than the triacylglycerol fraction. In contrast, the triacylglycerols contained a greater percentage of monounsaturated fatty acids (MUFA), especially oleic acid (18:1n-9). A remarkably high proportion of the free fatty acid fraction is DHA, approximately 26% of the FA in this fraction.

The fatty acid composition of TL in the abdominal wall, and of phospholipids and triacylglycerols of red muscle is also shown in Table 3. The profiles of total phospholipids and triacylglycerols in red muscle are very similar to those in white muscle. There was a higher percentage of DHA and 16:0 in WM phospholipids and more 18:0, 18:1n-7, 18:2n-6, 20:1n-9, 20:2n-6, 20:3n-6, and 20:4n-6 in RM phospholipids.

No pronounced differences of FA profiles among triacylglycerols of white muscle, triacylglycerols of red muscle, or TL of abdominal wall were observed. For some fatty acids there were significant differences among tissues; in particular, the AW total lipid contained slightly higher proportions of PUFA, which were characterized by high levels of MUFA, mainly oleic acid.

The total lipid composition of white muscle and abdominal wall differed substantially.

Discussion

The fatty acids in fillets from the common carp in this experiment are clearly affected by the lipid content of the fish. The samples from 2006 show that the cross BP was slightly leaner and therefore the n-3 PUFA content was increased. As all fishes were kept in the same environment and the feeding protocol was identical, it seems unlikely that this difference is caused by farming conditions. Therefore we speculate on possible metabolic differences. Early studies on fatty acids in carp were performed by Farkas and Csengeri (1976) and Farkas et al. (1978). The observed differences in n-3 PUFA in the fish in our experiment can possibly be ascribed to different fatty acid metabolism, because the lipid content was not significant. If this is the case, this cross might have higher Δ-5 and/or Δ-6 desaturase activity and possibly also a slightly higher β-oxidation.

In the study by Buchtová et al. (2007), no differences between crosses were observed when total lipids from fillet tissue samples were analyzed. However, it might be possible that breed effects are

Table 2 Fatty acid composition (%) of triacylglycerols and phospholipids from carp crosses ($n = 4$)

Fatty acid (%)	Triacylglycerols				Phospholipids			
	BL	PP	PL	BP	BL	PP	PL	BP
14:0	1.71 ± 0.16	1.69 ± 0.08	1.59 ± 0.09	1.66 ± 0.16	0.43 ± 0.04	0.43 ± 0.11	0.37 ± 0.08	0.38 ± 0.11
16:0	21.1 ± 0.59	21.8 ± 1.57	20.6 ± 1.18	20.9 ± 0.97	20.1 ± 1.74	19.2 ± 0.79	18.0 ± 1.01	18.6 ± 1.22
16:1n-7	8.41 ± 0.99	9.12 ± 0.98	7.75 ± 1.07	8.31 ± 1.00	3.57 ± 0.16	4.20 ± 1.06	3.35 ± 0.18	3.46 ± 0.70
18:0	5.23 ± 0.23 a	5.12 ± 0.46 a	5.88 ± 0.44 b	4.81 ± 0.27 a	7.06 ± 0.70	7.09 ± 0.71	7.35 ± 0.81	6.66 ± 0.45
18:1n-9	32.8 ± 1.86	31.3 ± 4.27	36.0 ± 4.36	29.5 ± 1.14	13.1 ± 0.30	13.5 ± 2.50	16.6 ± 1.30	11.4 ± 0.47
18:1n-7	3.41 ± 0.43	3.26 ± 0.08	3.31 ± 0.30	3.50 ± 0.17	2.97 ± 0.21	3.17 ± 0.28	3.04 ± 0.52	3.15 ± 0.17
18:2n-6	11.5 ± 3.14	10.4 ± 2.38	10.7 ± 3.35	14.1 ± 2.00	6.16 ± 0.97	6.72 ± 1.34	6.31 ± 0.99	6.77 ± 0.93
18:3n-3	3.53 ± 0.55	3.83 ± 0.55	3.08 ± 0.73	4.16 ± 0.33	1.04 ± 0.15	1.19 ± 0.11	1.07 ± 0.12	1.13 ± 0.07
20:1n-9	1.60 ± 0.07	1.47 ± 0.13	1.75 ± 0.23	1.54 ± 0.21	1.27 ± 0.21	1.41 ± 0.23	1.46 ± 0.45	1.17 ± 0.18
20:2n-6	0.33 ± 0.06	0.32 ± 0.11	0.37 ± 0.12	0.44 ± 0.07	0.48 ± 0.03	0.49 ± 0.12	0.54 ± 0.08	0.63 ± 0.06
20:3n-6	0.30 ± 0.06	0.27 ± 0.07	0.25 ± 0.04	0.30 ± 0.03	1.13 ± 0.07	1.18 ± 0.05	1.13 ± 0.05	1.08 ± 0.12
20:4n-6	0.65 ± 0.07	0.67 ± 0.10	0.62 ± 0.11	0.70 ± 0.01	7.47 ± 0.37	7.36 ± 0.83	7.35 ± 0.15	7.18 ± 0.44
20:4n-3	0.28 ± 0.17	0.42 ± 0.08	0.25 ± 0.08	0.39 ± 0.11	0.50 ± 0.13	0.54 ± 0.02	0.56 ± 0.05	0.56 ± 0.07
20:5n-3	1.49 ± 0.47	1.85 ± 0.15	1.34 ± 0.32	1.72 ± 0.37	6.51 ± 1.06	6.85 ± 0.27	6.67 ± 0.61	7.35 ± 0.49
24:0	0.02 ± 0.03	0.07 ± 0.05	0.03 ± 0.04	0.03 ± 0.04	0.59 ± 0.10	0.64 ± 0.16	0.73 ± 0.03	0.72 ± 0.12
22:5n-3	0.38 ± 0.16	0.51 ± 0.12	0.32 ± 0.12	0.34 ± 0.08	2.94 ± 0.71	2.99 ± 0.38	3.57 ± 0.30	3.45 ± 0.49
22:6n-3	0.58 ± 0.17	0.77 ± 0.22	0.50 ± 0.16	0.55 ± 0.14	13.4 ± 0.63	11.7 ± 2.10	12.7 ± 1.46	14.1 ± 0.97
ΣSFA	28.0 ± 0.70	28.7 ± 1.90	28.1 ± 1.40	27.5 ± 0.90	28.2 ± 1.42	27.3 ± 0.58	26.4 ± 0.74	26.3 ± 0.80
ΣMUFA	46.2 ± 2.18	45.1 ± 4.98	48.8 ± 5.51	42.9 ± 0.80	20.9 ± 0.57	22.3 ± 3.65	21.5 ± 2.21	19.2 ± 0.89
ΣPUFA	19.0 ± 2.12	19.0 ± 3.52	17.4 ± 4.56	22.7 ± 1.65	39.6 ± 0.97	39.0 ± 3.86	39.9 ± 1.18	42.2 ± 2.01
Σn-3 PUFA	6.27 ± 1.47	7.37 ± 1.09	5.48 ± 1.23	7.17 ± 0.96	24.4 ± 2.02	23.3 ± 2.29	24.6 ± 0.86	26.6 ± 1.46
Σn-6 PUFA	12.8 ± 3.20	11.7 ± 2.63	11.9 ± 3.55	15.5 ± 2.08	15.2 ± 1.13	15.8 ± 2.16	15.3 ± 1.05	15.7 ± 1.32
Σn-3/Σn-6	0.53 ± 0.20	0.65 ± 0.09	0.47 ± 0.09	0.47 ± 0.12	1.61 ± 0.24	1.49 ± 0.16	1.61 ± 0.14	1.70 ± 0.16

Different letters within the same lipid class indicate significant difference ($P < 0.05$) between the crosses

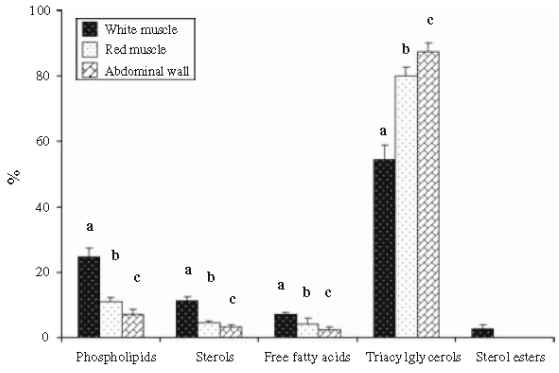

Fig. 3 Composition of lipid classes (percentage of total lipid) in different parts of common carp muscle ($n = 6$), mean ± standard deviation, different letters show significant differences ($P < 0.05$) among the three tissues within the same lipid class

only expressed in the polar or neutral lipid fractions which therefore should be analyzed separately. In our study the fatty acid composition of the phospholipids and triacylglycerols in the lipids extracted from different types muscle tissue from different crosses were analyzed and compared. The lack of differences between the fatty acid composition of the phospholipid and triacylglycerol fractions from the mirror carp crosses confirmed there was no significant difference.

In 2007, interesting results were obtained from the different tissues in this study. We found a high proportion of free fatty acids, especially in white muscle. There was also a high level of DHA in this fraction. In WM an increase in FFA with a decrease in phospholipids was seen. Several explanations can be suggested as underlying rationales for this result. The first is high metabolic activity of the fish, suggesting it can be attributed to higher enzymatic activity (phospholipase A_2) and de-novo synthesis of phospholipids or high conversion (desaturation) of n-3 PUFA to DHA (reviewed by Tocher 2003). Second,

Table 3 Fatty acid composition (%) of lipid classes and total lipids in white and red muscle and abdominal wall

Fatty acid (%)	White muscle				Red muscle		Abdominal wall
	Phospholipids ($n = 6$)	Triacylglycerols ($n = 6$)	Free fatty acids ($n = 4$)	Total lipids ($n = 6$)	Phospholipids ($n = 6$)	Triacylglycerols ($n = 6$)	Total lipids ($n = 6$)
14:0	0.35 ± 0.09 a	1.18 ± 0.10 x	1.04 ± 0.29	1.00 ± 0.06 c	0.26 ± 0.14 a	1.24 ± 0.12 x	1.47 ± 0.06 d
16:0	22.9 ± 0,58 a	22.2 ± 0.91 x	15.5 ± 4.10	22.1 ± 0.78 c	17.7 ± 1.61 b	22.4 ± 1.11 x	22.3 ± 0.81 c
16:1n-7	5.46 ± 0.72 a	10.7 ± 0.86 x	8.35 ± 0.64	9.33 ± 1.46 c	4.51 ± 1.06 a	10.6 ± 0.83 x	11.3 ± 0.91 d
18:0	7.62 ± 0.39 a	5.98 ± 0.53 x	4.45 ± 1.18	5.62 ± 0.54 c	10.1 ± 1.27 b	5.77 ± 0.28 x	5.91 ± 0.65 c
18:1n-9	19.9 ± 1.11 a	45.4 ± 1.17 x	26.4 ± 4.45	36.7 ± 2.80 c	18.9 ± 2.76 a	43.7 ± 0.54 y	41.4 ± 0.73 d
18:1n-7	3.22 ± 0.36 a	3.20 ± 0.29 x	2.73 ± 0.58	2.94 ± 0.40 c	4.14 ± 0.56 b	3.16 ± 0.30 x	3.24 ± 0.33 c
18:2n-6	4.88 ± 0.66 a	6.41 ± 0.76 x	5.24 ± 1.26	5.90 ± 0.95 c	6.66 ± 1.40 b	7.02 ± 0.87 x	7.38 ± 0.85 d
18:3n-3	0.65 ± 0.10 a	1.17 ± 0.13 x	1.03 ± 0.28	1.10 ± 0.17 c	0.81 ± 0.31 a	1.52 ± 0.23 y	1.93 ± 0.23 d
20:1n-9	2.31 ± 0.37 a	1.79 ± 0.11 x	1.62 ± 0.66	1.82 ± 0.05 c	3.50 ± 0.79 b	1.78 ± 0.13 x	1.66 ± 0.09 d
20:2n-6	1.16 ± 1.00 a	0.26 ± 0.19 x	0.84 ± 0.26	0.89 ± 0.76 c	3.53 ± 1.16 b	0.42 ± 0.07 y	0.43 ± 0.06 d
20:3n-6	1.35 ± 0.12 a	0.19 ± 0.03 x	0.61 ± 0.12	0.59 ± 0.09 c	2.10 ± 0.33 b	0.21 ± 0.02 x	0.24 ± 0.03 d
20:4n-6	7.78 ± 0.68 a	0.40 ± 0.07 x	2.18 ± 0.87	2.70 ± 0.78 c	9.72 ± 0.85 b	0.48 ± 0.03 y	0.60 ± 0.03 d
20:4n-3	0.44 ± 0.05 a	0.09 ± 0.03 x	0.30 ± 0.09	0.23 ± 0.04 c	0.34 ± 0.17 a	0.16 ± 0.03 y	0.23 ± 0.06 c
20:5n-3	5.21 ± 0.45 a	0.55 ± 0.06 x	1.71 ± 0.98	2.39 ± 0.64 c	4.61 ± 0.62 a	0.85 ± 0.11 y	1.22 ± 0.21 d
24:0	0.71 ± 0.19 a	–	0.31 ± 0.09	0.23 ± 0.08 c	0.85 ± 0.20 a	–	– d
22:5n-3	2.77 ± 0.25 a	0.14 ± 0.02 x	1.20 ± 0.45	1.16 ± 0.32 c	2.64 ± 0.65 a	0.21 ± 0.04 y	0.24 ± 0.07 d
22:6n-3	13.1 ± 1.92 a	0.27 ± 0.04 x	26.4 ± 14.7	5.26 ± 1.38 c	9.60 ± 2.17 b	0.37 ± 0.08 y	0.40 ± 0.15 d
ΣSFA	31.6 ± 0.47 a	29.3 ± 1.27 x	21.3 ± 5.52	29.0 ± 1.19 c	28.9 ± 1.49 b	29.4 ± 1.33 x	29.7 ± 1.34 c
ΣMUFA	30.9 ± 1.96 a	61.2 ± 1.40 x	39.1 ± 5.68	50.8 ± 3.56 c	31.1 ± 3.42 a	59.4 ± 1.17 x	57.6 ± 1.35 d
ΣPUFA	37.4 ± 2.19 a	9.48 ± 0.99 x	39.5 ± 11.0	20.2 ± 2.54 c	40.0 ± 2.93 a	11.2 ± 1.11 y	12.7 ± 1.15 d
Σn-3 PUFA	22.2 ± 1.86 a	2.21 ± 0.21 x	30.7 ± 13.0	10.2 ± 2.03 c	17.9 ± 3.34 b	3.11 ± 0.43 y	4.02 ± 0.66 d
Σn-6 PUFA	15.2 ± 1.31 a	7.27 ± 0.81 x	8.88 ± 2.18	10.1 ± 0.54 c	22.0 ± 1.30 b	8.14 ± 0.83 x	8.66 ± 0.85 c
Σn-3/ Σn-6	1.46 ± 0.18 a	0.31 ± 0.02 x	3.45 ± 2.57	1.01 ± 0.20 c	0.83 ± 0.20 b	0.38 ± 0.05 y	0.47 ± 0.09 d

Different letters within the same lipid class indicate significant difference ($P < 0.05$) between the tissues; phospholipids a–b, total lipid c–d, triacylglycerols x–y. The abbreviations are explained in the caption to Fig. 1

this high enzymatic activity can lead to a higher lipolysis post mortem, causing fatty acids to be released from the phospholipids as the total proportion of total phospholipids decreases when FFA increase (Huss 1988).

The most important factor influencing the fatty acid composition of the fish was the lipid content. The white muscle lipid content in 2006 was higher than that in 2007, which was because less of the sample consisted of red muscle, which contains much more fat.

It is known that phospholipids serve as components of cell membranes and structures whereas triacylglycerols serve as an energy reserve and are mainly stored in adipose tissues (Sargent et al. 1995). Therefore the white muscle, being the leanest, with a low lipid content (~1%), contains a significantly higher proportion of n-3 HUFA, 20:5n-3, 22:5n-3, and 22:6n-3. FA composition in TL was strongly affected by the FA composition of total phospholipids. Similar findings were reported by Kiessling et al. (2001) in studies of rainbow trout (*Oncorhynchus mykiss*). The relative proportion of total phospholipids and triacylglycerols was highly dependent on 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 on FA composition within the TL. There were

significant differences between the FA composition of the dorsal and ventral parts of the different species (Testi et al. 2006).

The origin of the n-3 HUFA is most likely the plankton, which is abundant in the ponds (Přikryl 1984; Faina 1983; Janeček and Přikryl 1982). Plankton samples all contain a higher percentage of n-3 PUFA and a higher ratio of n-3 to n-6 than fish tissue. It is likely that the fatty acid composition does not change substantially because the fish lipid profile will mostly mirror the late summer–autumn composition of the feed, including the plankton which is available in the ponds. In the study by Linko et al. (1992), no large variation of total n-3 PUFA and HUFA was observed. It is, therefore, plausible that the origin of the large amount of n-3 HUFA in the carp muscle is of plankton origin.

Supplementation with wheat causes fattening of the carp by FA synthesis de novo and an increase in the growth of fatty tissue. In addition, wheat contains n-6 PUFA, which seem to be stored in the flesh lipid stores to a large extent, possibly as a result of small amounts of n-3 PUFA in the feed.

These results from carp show the abdominal wall has a high lipid content (33.5%) dominated by triacylglycerols. In contrast with the low level of oleic acid in plankton, which is one part of the feed, the high level of oleic acid in the triacylglycerols of this tissue most likely originates from the supplemental cereal feeding. With regard to human nutrition, this tissue and, in particular, the storage triacylglycerol fraction have less favourable ratios of polyunsaturated n-3/n-6 fatty acids to total phospholipids. We can argue that this low proportion of PUFA n-3 can be improved by changing the feed composition to other vegetable additives rather than cereals.

We conclude that the common carp has the potential to become an attractive fish in terms of lipid content and composition. The lipids in the carp currently produced are of high nutritive value, especially in the lean parts of the fillets, relative to the amount of n-3 PUFA in the total diet of central Europe. In addition, improvement of the lipid composition of carp cultured in traditional pond production should be feasible by changing supplementation by wheat feeding to other crops with less n-6 FA and less carbohydrate.

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Chapter 3

Sesamin as a potential modulator of fatty acid composition in common carp (*Cyprinus carpio*)

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Záhlaví liché
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Záhlaví sudé
Chapter 3

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Sesamin as a potential modulator of fatty acid composition in common carp (*Cyprinus carpio*)

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Abstract

Sesamin enhances the DHA content of tissue lipids in salmonid fish. We have examined whether this effect also occurs in common carp (*Cyprinus carpio*). Two-year-old common carps were fed a diet containing 5.8 g sesamin kg^{-1} feed for 69 days. No significant differences were found in the fish weight, specific growth rate, food conversion ratio and lipid content in white muscle. The experimental diet with sesamin increased the percentage of 18:3 n-3 and decreased 18:4 n-3, 22:5 n-3, 22:6 n-3 as well as the desaturation index reflecting the ratio of n-3 long-chain polyunsaturated fatty acids/18:3 n-3 in the triacylglycerol fraction. Sesamin increased the total cytochrome P450 content in hepatopancreatic microsomes as well as 7-ethoxyresorufin *O*-deethylase (EROD) activity. However, a detailed microarray analysis using 26K gene probes failed to establish any significant pattern of transcriptional response, including lipid biosynthetic genes. Together, these results indicate that under conditions of our study, sesamin was ineffective in the common carp as a means of achieving the changes in the tissue lipid composition as were seen in salmonids.

Keywords: cytochrome P450, EROD, linolenic acid, microarray, sesamin, transcriptomics

Introduction

Fish are the major source of n-3 long-chain polyunsaturated fatty acids (n-3 LC PUFA) in the human diet (Sargent, Henderson & Tocher 1989); n-3 fatty acids (FA) and n-3 LC PUFA are especially important for maintaining cardiovascular and mental health (Simopoulos 1991; Mozaffarian & Rimm 2006). The major source of these FA for aquaculture production is fish oil from marine pelagic fish. As aquaculture is increasing, there is a growing need for fish oil for fish feed production. This situation is in general solved by the partial replacement of fish oil by vegetable oils; however, this is causing a decrease in n-3 LC PUFA in farmed fish (Pickova & Morkore 2007). There is an urgent need to investigate and develop new strategies for fish oil replacement that will ensure high levels of beneficial n-3 LC PUFA for human consumption.

A finishing diet strategy has been developed as one of the possible solutions. Vegetable oil-based diets used during the fish grow-out phase are replaced by fish oil-based diets during the last few months before slaughtering. This technique ensures a beneficial FA composition, together with the effective utilization of n-3 LC PUFA (reviewed by Turchini, Torstensen & Ng 2009). Recently, alternative oil sources have been identified and tested in aquafeeds. For example, docosahexaenoic acid (DHA)-rich oils derived from single-cell microalgae (Ganuza, Benítez-Santana, Atalah, Vega-Orellana, Ganga & Izquierdo 2008), bacteria, fungi and thraustochytrids (Bajpaj & Bajpaj 1993; Hinzpeter, Shene & Masson 2006) or calanoid copepods (Olsen, Henderson, Sountama, Hemre, Ringo, Melle & Tocher 2004) were studied. However, these alternatives have high production costs and are not commonly used in aquaculture production. Genetically modified, GMO, n-3 LC PUFA-enriched

oil-seeds are showing promising results (Robert 2006) but consumers might dislike the use of GMOs foods.

An alternative approach might be the use of biologically active compounds in fish feeds, which can affect the fish metabolism to synthesize more n-3 LC PUFA from α-linolenic acid (ALA). Trattner, Kamal-Eldin, Brännäs, Moazzami, Zlabek, Larsson, Ruyter, Gjoen and Pickova (2008) suggested that sesamin could be such a compound. Sesamin is the major oil-soluble lignan found in sesame seed (Moazzami & Kamal-Eldin 2006) and has been shown to have several effects on lipid metabolism in mammalian systems (Fujiyama-Fujiwara, Umeda-Sawada, Kuzuyama & Igarashi 1995; Mizukuchi, Umeda-Sawada & Igarashi 2003).

As a first study investigating sesamin effects in fish, Trattner, Kamal-Eldin *et al.* (2008) found that sesamin/episesamin supplementation increases the level of DHA up to 37% in white muscle of rainbow trout (*Oncorhynchus mykiss*) fed with high-ALA vegetable oil. An *in vitro* study with Atlantic salmon (*Salmo salar*) hepatocytes (Trattner, Ruyter, Ostbye, Gjoen, Zlabek, Kamal-Eldin & Pickova 2008) 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.

Common carp (*Cyprinus carpio*) is one of the most important fish species in aquaculture. Carps are normally fed with diets that do not include fish oil (Tacon & Metian 2008). They are traditionally reared in earthen ponds and their diet is based on natural food in the ponds, usually with cereal supplementation. As cereals are rich in carbohydrates and have very low levels of n-3 FA, the flesh of farmed carps generally contains a high level of oleic acid and a low level of favourable n-3 LC PUFA (Csengeri 1996). There is interest, if possible, to increase the n-3 LC PUFA content in carp fillet. Hence, the aim of this study is to see whether sesamin (as shown in salmonids) can modulate FA metabolism and possibly increase the final n-3 LC PUFA content of common carp fillet.

Materials and methods

Diet composition

As a basal diet, a slightly modified commercial feed, DAN-EX 1344 (BioMar A/S, Brande, Denmark), was prepared by adding a mixture of linseed oil: rapeseed oil (3:2 v/v). The diet was formulated to contain 440 g kg^{-1} protein, 239 g kg^{-1} carbohydrates and 110 g kg^{-1} lipids. The experimental diet was prepared by adding sesamin 5.8 g kg^{-1} feed. Sesamin (purity 98%) was obtained from Kebiotech (Beijing, China). The lipid content and FA composition of the experimental diets are shown in Table 1.

Table 1 The lipid content and fatty acid composition (%) of the experimental diets (mean ± SD), (n = 4/group)

	Control diet	Diet with sesamin
Lipid content	10.9 ± 0.5	11.5 ± 1.2
SAFA	13.5 ± 0.3	14.7 ± 1.9
MUFA	26.8 ± 0.5	26.4 ± 1.2
18:2 n-6	19.4 ± 0.2	18.9 ± 0.9
18:3 n-3	28.2 ± 0.3	27.2 ± 1.4
20:5 n-3	3.5 ± 0.2	3.9 ± 0.4
22:5 n-3	0.5 ± 0.0	0.5 ± 0.1
22:6 n-3	3.0 ± 0.1	3.3 ± 0.4
Σ n-3/Σ n-6	1.8 ± 0.0	1.8 ± 0.1

SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids.

Fish and rearing facilities

Two-year-old common carp (n = 60; crossbreed Tata carp × Ropsha carp) raised in an earthen pond and with a mean live weight of 730 g were transported to the experimental facility of Research Institute of Fish Culture and Hydrobiology at Vodnany (Czech Republic). The fish were divided into six groups of 10 animals and placed in six 1 m^3 tanks. Each tank was supplied with well-aerated water from one recirculation system after mechanical and biological filtering (flow rate 0.25 L s^{-1}; oxygen concentration ranged from 7 to 10 mg L^{-1}; and water temperature 21–24 °C). The water level in the tanks was set to 0.7 m, which yielded a water volume of 700 L. Fish in all the tanks were exposed to a constant 12 h:12 h light/dark cycle. During the 3-week adaptation period before the start of the experiment, all fish were fed the basal diet with a feed ration equivalent to 1.5% of the biomass. The feed was supplied to the tanks for 9 daylight hours using mechanical belt-feeders (BFS12; Aquatic Eco-systems, Apopka, FL, USA). Two days before the start of the actual feeding experiment, four fish were removed to achieve the experimental stocking density of six fish per tank and one carp was randomly selected from each tank for analyses of the initial muscle FA composition (Table 2).

Table 2 Initial carp white muscle fatty acid composition

	Phospholipids	Triacylglycerols
SAFA	32.1 ± 1.69	25.9 ± 0.51
MUFA	25.1 ± 1.49	56.3 ± 2.78
18:2 n-6	4.6 ± 0.46	11.4 ± 1.14
18:3 n-3	0.5 ± 0.14	1.4 ± 0.43
18:4 n-3	ND	0.5 ± 0.12
20:2 n-6	0.6 ± 0.07	0.3 ± 0.03
20:3 n-6	1.2 ± 0.14	0.2 ± 0.02
20:4 n-6	8.7 ± 0.93	0.5 ± 0.07
20:5 n-3	5.1 ± 1.02	1.3 ± 0.53
22:4 n-6	1.1 ± 0.22	ND
22:5 n-3	3.4 ± 0.53	0.5 ± 0.16
22:6 n-3	17.7 ± 1.61	1.7 ± 0.78

Data are expressed as percentage of identified fatty acids (mean ± SD), ($n = 6$).
For abbreviations, see Table 1; ND, not detected.
Lipid content 0.96 ± 0.30%.

The experiment lasted until the fish doubled their weight after 9 weeks of feeding. The two different diets were fed to fish kept in a randomized set of tanks (three tanks with the control diet without sesamin and three tanks with the experimental diet with sesamin). Feed rations were equivalent to 1.5–2% of biomass. Each fish was weighed individually at the start, after 21, 42 days and at the end of the 9-week feeding period. The specific growth rate (SGR) and the feed conversion ratio (FCR) were calculated according to the following equations:

$$\mathrm{SGR} = [(\ln W_t - \ln W_o) \times t^{-1}] \times 100$$

$$\mathrm{FCR} = F \times (W_t - W_o)^{-1}$$

where W_t is the final weight, W_o is the initial weight, t is the number of days and F is the amount of ingested feed.

At the end of the feeding experiment, fish were starved for 1 day before the final sampling. No mortality was observed during the adaptation and experimental period. Before killing, all fish were anaesthetized (clove oil 0.033 mL L^{-1}).

All fish were sampled for further analyses ($\sim$ 5 g of white dorsal muscle and hepatopancreas). Samples were immediately frozen in liquid nitrogen and stored at − 80 °C before analyses.

Lipid analyses

Lipid analyses of tissues and feed samples were performed as described previously by Mráz and Pickova (2009). The samples were extracted with hexane–isopropanol (Hara & Radin 1978). Total lipids were fractionated by thin-layer chromatography for the separation of lipid classes in accordance to Pickova, Dutta, Larsson and Kiessling (1997). Fatty acids were methylated (Appelqvist 1968) and analysed using a gas chromatograph (Varian CP3800, Stockholm, Sweden) equipped with a flame ionization detector and a split injector and fitted with a 50 m length × 0.22 mm i.d. × 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 of the respective retention times with the standard mixture GLC-461 (Nu-check Prep, Elysian, MN, USA). Peak areas were integrated using STAR CHROMATOGRAPHY workstation software version 5.5 (Varian AB). Fatty acids were quantified using the internal standard methyl 15-methylheptadecanoate (Larodan Fine Chemicals AB, Malmo, Sweden).

Sesamin analyses

For the analysis of sesamin, the lipid extracts of white dorsal muscle and feed were dissolved in hexane and analysed using high-performance liquid chromatography (HPLC) as described by Moazzami and Kamal-Eldin (2006). High-performance liquid chromatography was performed using a Bischoff HPLC pump (Bischoff Analysentechnik und Geräte GmbH, Leonberg, Germany), an Alltech SI 5U silica column (4.6 × 250 mm; Alltech Associates, Deerfield, IL, USA) and CHROMELEON software (Version 4.12; Gynkotek HPLC, Mahwah, NJ, USA). The mobile phase was hexane/1,4-dioxane (94:4, by vol). The fluorescence detector was operated at an excitation wavelength of 296 nm and an emission wavelength of 324 nm. External standards were used for identification and quantification.

Preparation of liver microsomes and determination of the total cytochrome P450 content

The microsomal fraction was prepared from the hepatopancreatic homogenate using the Ca-aggregation method as described by Zamaratskaia, Zlabek, Chen and Madej (2009), with minor modifications. Briefly, 1.5 g of frozen hepatopancreatic tissue was homogenized with 3 mL of ice-cold 10 mM Tris-HCl buffer containing 250 mM sucrose at pH 7.4. The

homogenate was centrifuged at 10 000 *g* for 10 min at 4 °C to separate the mitochondria from the post-mitochondrial fraction. Calcium chloride was added to the supernatant to yield a final concentration of 8 mM, well mixed and allowed to stand at 4 °C for 4 min. Then, the supernatants were centrifuged at 25 000 *g* for 30 min at 4 °C to separate the microsomal and cytosolic fractions. The resulting pellet of microsomes was resuspended in 50 mM Tris-HCl containing 0.1 mM EDTA and 20% glycerol at pH 7.4. The microsomal protein was assayed using a commercially available kit (Bio-Rad laboratories, Hercules, CA, USA) according to the manufacturer's instructions, using bovine serum albumin (BSA) as a standard. Samples were stored in small aliquots at − 80 °C until required for assay. Enzyme activity assays were performed within 1 month of microsomal preparations.

The total cytochrome P450 content in liver microsomes was determined using the spectrophotometric method of Omura and Sato (1964), measuring the differences in the spectra (dithionite+carbon monoxide) − dithionite.

Microsomal 7-ethoxyresorufin *O*-deethylase (EROD) activity

The activities of EROD were estimated using an HPLC-based method validated recently for routine determination of EROD activities in porcine liver microsomes (Zamaratskaia & Zlabek 2009) and conveniently adapted for fish microsomes. After incubation at 25 °C, 0.5 mL of cold methanol was added to stop the reaction. The reaction mixture was centrifuged at 7500 *g* at 4 °C for 5 min, and the amount of resorufin formed in the resulting supernatant was measured using HPLC with a fluorescence detector (excitation and emission wavelengths of 560 and 586 nm respectively) (Zamaratskaia & Zlabek 2009).

The assay for enzymatic activity was performed under linear conditions of metabolite formation with regard to the incubation time and protein concentrations. Enzymatic activities were expressed as pmol of resorufin formed per min and per mg of microsomal proteins.

The HPLC analyses were performed on a system consisting of a pump (L-7100), an autosampler (L-7200), a fluorescence detector (L-7485) or a UV-VIS detector (L-4250) and D-7000 HPLC MANAGER software (Merck, Hitachi, Tokyo, Japan). A Hypersil ODS (3 μm, 60 × 4.6 mm) with a guard column was used for the quantification of resorufin.

Microarray analysis

To identify differences in the gene expression, profiles of liver tissue from control (C) and sesamin-treated (S) carp, fish from each group were maintained in three identical tanks. The experimental design included a three-vertex-loop design for each group with links between the two blocks (Fig. 1). This comprised 18 dual channel microarrays, six for the control block, six for the treatment group and six for the links between the two conditions. Experimental tank comparisons were carried out in duplicate with dye flip. Where possible, tissues from male and female animals were compared. The design was fully balanced, with the exceptions of the C2 vs. C3 and the C3 vs. S3 comparisons.

Total RNA extraction, target preparation and microarray hybridization

Samples of 0.5 g of fresh hepatopancreatic tissue were disrupted in 5 mL of TriReagent using an Ultra-Turrax homogenizer (IKA, Staufen, Germany), and immediately frozen in liquid nitrogen for storage. Total RNA was extracted 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) and reliability was ascertained using an Agilent RNA 6000 NANO KIT (Agilent Technologies, Waldbronn, Germany) with an Agilent 2100 Bioanalyzer. Fluorescently labelled cDNA was generated from

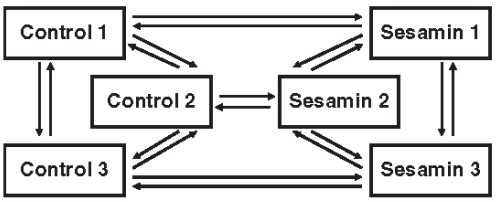

Figure 1 Microarray experiment: design. This experimental design compared liver cDNA samples from three different replicates control and sesamin-treated carp. It comprised 18 microarrays represented by the arrows, the tail indicating a Cy3 and the head a Cy5 labelled cDNA preparation, the total number of samples being 36. Each sample for a given treatment group represents a different cDNA preparation, such that each was determined by a total of six replicate preparations.

10 μg of total RNA per sample using the SuperScript Plus Indirect cDNA Labeling System (Invitrogen L1014-04, -05 and -06). Dye-labelled cDNA was used for microarray hybridization. Two samples of dye-labelled cDNA to be co-hybridized were combined and dried down to completion by centrifugation under vacuum. Eighteen 26K common carp cDNA microarrays (carpARRAY ver5; Williams, Li, Hughes, Gonzalez, Vernon, Vidal, Jeney, Jeney, Dixon, McAndrew, Bartfai, Orban, Trudeau, Rogers, Matthews, Fraser, Gracey & Cossins 2008) printed on Corning Gap II slides were pre-hybridized at 50 °C for 60 min in a pre-hybridization solution consisting of 3 × saline sodium citrate (SSC), 0.1% sodium dodecyl sulphate (SDS) and 0.1 mg/mL BSA. For hybridization, each dried dye-labelled cDNA was mixed with 25 μL of molecular biology-grade water and 25 μL of pre-heated 2 × hybridization buffer. The hybridization buffer, consisting of 6 × SSC, 0.2% SDS and 0.2 mg mL ^{1} BSA, was heated to 50 °C. The dye-labelled target was then denatured at 100 °C for 2 min in the dark and further incubated at room temperature for 1 min. Before hybridization, all targets were briefly centrifuged to collect condensation and incubated at 50 °C in the dark until the arrays were ready to be loaded. Hybridizations were performed in a Maui hybridization system (BioMicro Systems, Salt Lake City, UT, USA). Maui mixers were adhered to the arrays, loaded with 45 μL of the target and incubated for 16 h in the hybridization system at 50 °C. Slides were then washed once with 2 × SSC, 0.1% SDS for 5 min; twice in 0.1 × SSC, 0.1% SDS for 5 min; once in 0.1 × SSC for 1 min; twice in 0.1 × SSC for 2 min; once in 0.01 × SSC for 30 s; and once in 0.01 × SSC for 30 s. All wash solutions were pre-heated to 42 °C before post-hybridization washing. Arrays were scanned at 100% PMT voltages and at a resolution of 10 μm using an Agilent DNA microarray scanner. BLUEFUSE software (BlueGnome, Great Shelford, Cambridge, UK) was then used for background correction and raw data collection.

Statistical analyses

Data are shown as mean ± SD. The significance ($P < 0.05$) between treatments was tested using the one-tail Students' *t*-test.

The microarray data were normalized using VSN (Huber, Von Heydebreck, Sültmann, Poustka & Vingron 2002) and the 'loess- based dye-bias and spatial bias correction method (Cleveland & Devlin 1988). The data were then represented by a linear model, with model parameters being estimated using the maximum likelihood estimation (MLE) approach. The parameters were presented as the log-ratio of gene expressions for the contrast of the treatment with the control groups of fish, which was visualized using *P*-value histograms and a 'volcano' plot. The differentially expressed (DE) genes were extracted using *Q*-values (Storey 2002) and the control false discovery rate (FDR) at the level of 10% (Benjamini & Hochberg 1995, 2000). Then, taking all known genes on the array as a reference gene set and the up- or down-regulated DE genes as a query gene set, we used a hyper-geometric test to ascertain whether any gene ontology (GO) terms were associated with sesamin treatment. Significant GO terms were extracted using threshold *Q*-values = 0.10 and we report only daughter terms as recommended (Rhee, Wood, Dolinski & Draghici 2008). In order to explore in greater detail the potential responses of coherent groups of genes, we have developed a new test method that is based on the rank of the *P*-values for the subset of genes in question (i.e. those belonging to a GO term). We used the mean of the rank as a test statistic for the GO term relative to that for all genes.

Results

Fish performance

After 69 days of the experiment, no statistically significant differences were found in the fish weight, SGR and food conversion ratio (Table 3; $P > 0.05$). No mortality was observed during the experiment.

Lipid and sesamin analyses

The white muscle lipid content (%) was slightly higher in the sesamin group (2.42 ± 0.96) compared with the control group (2.10 ± 1.09) but this difference did not reach statistical significance ($P > 0.05$). Some of the FA in the white muscle were significantly affected

Table 3 Final weight, survival, food conversion ratio and specific growth rate of fish fed two different diets: Control, fish fed the control diet; Sesamin, fish fed diet with sesamin

	Control	Sesamin
Initial weight (g)[1]	830 ± 128.8	832 ± 110.8
Final weight (g)[1]	1661 ± 316.6	1726 ± 279.7
FCR[2]	1.84 ± 0.09	1.73 ± 0.24
SGR (% day ^{1})[2]	1.00 ± 0.03	1.06 ± 0.11

Mean ± SD.
FCR, food conversion ratio; SGR, specific growth rate; no significant difference ($P > 0.05$); weight: [1]n = 18; [2]n = 3.

Table 4 White muscle phospholipid and triacylglycerol fatty acid composition from carp fed two different diets: Control, fish fed the control diet; Sesamin, fish fed diet with sesamin

	Phospholipids		Triacylglycerols	
	Control	Sesamin	Control	Sesamin
SAFA	27.6 ± 1.81	26.9 ± 1.12	21.7 ± 0.99	21.8 ± 0.78
MUFA	21.8 ± 2.12	21.7 ± 1.63	49.7 ± 1.63	48.7 ± 2.27
18:2 n-6	5.7 ± 0.54	5.5 ± 0.79	11.5 ± 0.85	11.8 ± 0.91
18:3 n-6	ND	ND	0.2 ± 0.09	0.2 ± 0.12
18:3 n-3	2.9 ± 0.34	3.2 ± 0.36	9.8 ± 1.01*	10.7 ± 1.18*
18:4 n-3	0.3 ± 0.07	0.2 ± 0.08	0.7 ± 0.06*	0.6 ± 0.07*
20:2 n-6	0.6 ± 0.07	0.6 ± 0.04	0.3 ± 0.04	0.3 ± 0.03
20:3 n-6	1.1 ± 0.13*	1.0 ± 0.06*	0.2 ± 0.03	0.2 ± 0 03
20:4 n-6	4.7 ± 0.65	4.7 ± 0.73	0.3 ± 0.09	0.3 ± 0.08
20:3 n-3	0.6 ± 0.07	0.6 ± 0.06	0.4 ± 0.06	0.4 ± 0.04
20:4 n-3	0.8 ± 0.11	0.8 ± 0.09	0.4 ± 0.06	0.4 ± 0.07
20:5 n-3	6.4 ± 0.58	6.4 ± 0.51	1.6 ± 0.19	1.7 ± 0.17
22:4 n-6	0.5 ± 0.08	0.5 ± 0.18	ND	ND
22:5 n-3	3.3 ± 0.29	3.4 ± 0.24	0.8 ± 0.09*	0.7 ± 0.08*
22:6 n-3	23.7 ± 1.76	24.4 ± 2.25	2.7 ± 0.31*	2.3 ± 0.32*
LC PUFA/18:3 n-3	11.8 ± 1.84	11.3 ± 1.71	0.59 ± 0.06*	0.52 ± 0.04*

For abbreviation, see Table 1; LC PUFA fatty acids with more than 18 carbons.
*Statistical difference ($P < 0.05$) between groups within the same lipid class (mean ± SD), ($n = 12$ in each group).
Data are expressed as percentage of identified fatty acids.
PUFA, poly unsaturated fatty acids; ND, not detected.

by the presence of sesamin in the feed (Table 4). In the sesamin group, 18:3 n-3 in the triacylglycerol fraction increased and 18:4 n-3, 22:5 n-3, 22:6 n-3 as well as the desaturation index n-3 LC PUFA/18:3 n-3 decreased. The level of 20:3 n-6 decreased in the phospholipid fraction after feeding the sesamin-enriched diet compared with the control group. The total lipid FA composition is presented in Table 5 as percentage and mg per 100 g of fillet.

Sesamin was detected in the white muscle of the sesamin-fed fish (0.57 ± 0.16 mg g ^{1} lipid).

Total content of cytochrome P450 and EROD activity

The total amount of cytochrome P450 was significantly higher ($P < 0.05$) in the sesamin group compared with the control (Fig. 2), with 0.138 ± 0.032 and 0.119 ± 0.027 nmol mg ^{1} microsomal protein respectively. Hepatopancreatic EROD activity was also higher ($P < 0.01$) in the sesamin group (30.0 ± 19.3 pmol min ^{1}mg ^{1}microsomal protein) compared with the control group (6.3 ± 3.7 pmol min ^{1}mg ^{1} microsomal protein) (Fig. 3).

Table 5 White muscle total lipid fatty acid composition from carp fed two different diets: Control, fish fed the control diet; Sesamin, fish fed diet with sesamin

	Total lipid (%)		Total lipid (mg/100g fillet)	
	Control	Sesamin	Control	Sesamin
SAFA	22.9 ± 0.97	22.7 ± 0.44	399 ± 219	511 ± 228
MUFA	42.2 ± 4.17	43.2 ± 3.60	779 ± 517	1005 ± 495
18:2 n-6	9.9 ± 0.97	10.6 ± 0.81	182 ± 121	246 ± 118
18:3 n-6	0.2 ± 0.07	0.1 ± 0.05	2 ± 1	2 ± 1
18:3 n-3	8.3 ± 1.38	9.7 ± 1.07	158 ± 116	189 ± 119
18:4 n-3	0.1 ± 0.05	0.2 ± 0.01	3 ± 2	4 ± 2
20:2 n-6	0.4 ± 0.08	0.3 ± 0.04	6 ± 2	7 ± 3
20:3 n-6	0.5 ± 0.15	0.3 ± 0.09	7 ± 2	7 ± 2
20:4 n-6	1.4 ± 0.73	1.1 ± 0.45	20 ± 5	21 ± 5
20:3 n-3	0.4 ± 0.07	0.4 ± 0.06	7 ± 4	9 ± 4
20:4 n-3	0.5 ± 0.04	0.5 ± 0.05	9 ± 5	12 ± 5
20:5 n-3	3.1 ± 0.83	2.8 ± 0.50	49 ± 16	58 ± 21
22:4 n-6	0.1 ± 0.10	0.1 ± 0.07	1 ± 1	1 ± 1
22:5 n-3	1.5 ± 0.40	1.2 ± 0.30	23 ± 7	26 ± 8
22:6 n-3	8.5 ± 3.21	6.6 ± 2.35	123 ± 25	126 ± 29

For abbreviation, see Table 1 (mean ± SD), ($n = 12$ in each group).
Data are expressed as percentage of identified fatty acids and total amount of fatty acids per 100 g of fillet.

Microarray detection of sesamin-regulated genes

We have compared the gene expression profiles of liver from sesamin-treated carp with control carp, using dual-colour microarrays and an interwoven loop design comprising 18 arrays and 36 RNA preparations. Figure 4a shows the distribution of

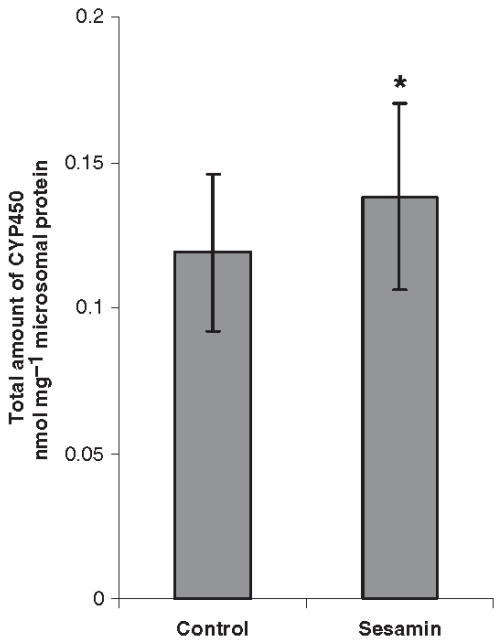

Figure 2 Total amount of cytochrome P450 in hepatopancreatic microsomes from carp fed two different diets: Control, fish fed control diet; Sesamin, fish fed diet with sesamin. Each bar represents the mean total amount of cytochrome P450 ± standard deviation ($n = 16$ in each group). *Significant difference ($P < 0.05$).

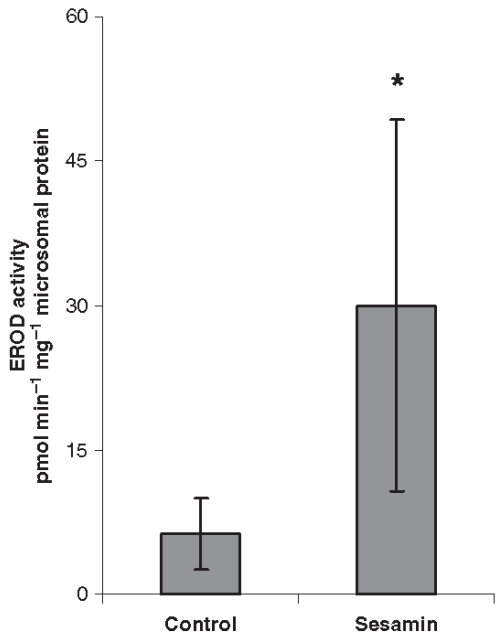

Figure 3 7-ethoxyresorufin *O*-deethylase (EROD) activity in hepatopancreatic microsomes from carp fed two different diets: Control, fish fed the control diet; Sesamin, fish fed diet with sesamin. Each bar represents the mean EROD activity ± standard deviation ($n = 18$ in each group). *Significant difference ($P < 0.01$).

P-values across all gene probes, this being the best means of estimating the false discovery rate (FDR) by extrapolating the random values at $P > 0.3$ across to those at $P < 0.05$. This suggests an acceptable FDR and that a low proportion of gene probes with low *P*-values were false positives. Figure 4b illustrates the corresponding 'volcano' plot, indicating the relationship between *P*-values and fold change across all gene probes. This shows that the *P*-values were comparatively high, and few gene probes displayed fold-change values above ~1.5-fold. Thus, as a whole, the gene responses were somewhat small, and not supported by low *P*-values. Nevertheless, based on an FDR of 0.1, that is, the proportion of false positives that was estimated at 10%, we extracted 166 significantly down-regulated and 496 up-regulated gene probes. By contrast, we were unable to detect any genes that were differentially expressed between the replicate tanks (data not shown), and so tanks effects were not contributing to the outcome.

Unique sesamin-sensitive genes were subjected to enrichment analysis using GO categories and a hypergeometric test statistic. This revealed just four categories of down-regulated genes and eight categories of up-regulated genes, of which only two of the latter involved intermediary metabolism (Table 6). These were the 'FA biosynthetic process' and the 'pentose phosphate shunt,' both of which were populated by triosephosphate isomerase 1a and 1b, and by two other zebrafish annotations, zgc:172186 and :92607 respectively. Enrichment analysis of the combined down- and up-regulated genes revealed no significant GO categories with FDR < 0.1. We have also developed a more sensitive, statistically based ranking test to explore whether groups of genes display patterns of regulation. This test is similar to the Gene Set Enrichment Analysis (GSEA). Table 7 lists the GO categories with FDR < 0.1, of which 'cholesterol efflux', 'response to oxidative stress' and 'lipoprotein metabolic process' bear some link with lipid metabolism. The two most responsive terms were 'translation' and 'translational elongation,' which included over 232 genes, most of which displayed a small up-regulation in sesamin-treated fish. Also, most genes of 'aerobic metabolism' displayed a slight down-regulation. Either way, these two tests provide little direct support for a major change in lipid metabolism following exposure to sesamin, at least at the level of gene transcription.

Discussion

The aim of this study was to investigate the effect of a diet supplemented with sesamin on lipid metabolism, total cytochrome P450 and microsomal EROD activity, which is commonly used as a marker for exposure to xenobiotic compounds in fish, and gene expression in common carp.

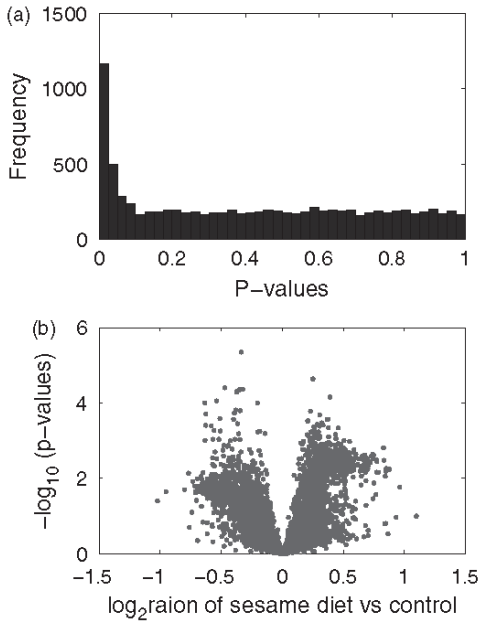

Figure 4 Analysis of differentially expressed carp gene probes based on the comparison of control and sesamin-treated carp. (a) The distribution of *P*-values across all microarray probes, with random *P*-values (b) volcano plot indicating the relationship between *P*-values and fold changes across all gene probes.

Similarly, as in rainbow trout (Trattner, Kamal-Eldin *et al.* 2008), we did not observe any effects of sesamin on the mortality, growth performance or muscle lipid content of reared carp. Unexpectedly, dietary sesamin did not alter positively the FA composition of triacylglycerols in the present study, whereas the percentages of docosahexaenoic acid in the white muscle phospholipid and triacylglycerol fraction of rainbow trout (Trattner, Kamal-Eldin *et al.* 2008) were higher in fish fed sesamin. Moreover, Trattner, Ruyter *et al.* (2008) demonstrated the same effect in an *in vitro* study on cultivated Atlantic salmon hepatocytes exposed to the presence of sesamin/episesamin.

There could be several explanations for the different responses of salmonids and cyprinids to sesamin treatment. (1) An evolutionary aspect should be considered. Salmonids as predators are not naturally consuming these types of vegetable substances, whereas cyprinids, being omnivores, usually encounter such compounds in their food. Kushiro, Takahashi and Ide (2004) found that there are species-dependent differences in the physiological response to dietary lignans. In rats, the sesamin/episesamin considerably increased the hepatic activity and the mRNA levels of enzymes involved in FA oxidation, while those enzymes involved in lipogenesis were strongly down-regulated. In contrast, lignans did not modify these variables in mice and hamsters. (2) It was shown that n-3 LC PUFA biosynthesis is regulated by nutritional factors. The

Table 6 The gene ontology (GO) categories displaying enrichment of up-regulated and down-regulated genes

GO accession	GO name	*P*-value	*Q*-value
Down-regulated genes			
GO:0006613	Cotranslational protein targeting to membrane	0.0002	0.0197
GO:0006825	Copper ion transport	0.0030	0.0852
GO:0006139	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.0032	0.0852
GO:0006066	Cellular alcohol metabolic process	0.0061	0.0973
GO:0045822	Negative regulation of heart contraction	0.0061	0.0973
Up-regulated genes			
GO:0001578	Microtubule bundle formation	0.0035	0.0675
GO:0006754	ATP biosynthetic process	0.0035	0.0675
GO:0007173	Epidermal growth factor receptor signalling pathway	0.0035	0.0675
GO:0007424	Open tracheal system development	0.0035	0.0675
GO:0048565	Gut development	0.0035	0.0675
GO:0006098	Pentose-phosphate shunt	0.0041	0.0675
GO:0006633	Fatty acid biosynthetic process	0.0062	0.0765
GO:0046034	ATP metabolic process	0.0062	0.0765

Table 7 Evaluation of significant gene ontology (GO) terms using the *p*-rank statistic

GO accession	GO name	*P*-value	*Q*-value	Number of genes
GO:0006414	Translational elongation	4.4E-10	1.0E-07	87
GO:0006412	Translation	2.4E-07	2.9E-05	145
GO:0051597	Response to methylmercury	6.2E-05	0.0042	29
GO:0009060	Aerobic respiration	7.1E-05	0.0042	28
GO:0046686	Response to cadmium ion	0.0003	0.0137	37
GO:0033344	Cholesterol efflux	0.0007	0.0262	15
GO:0006123	Mitochondrial electron transport, cytochrome *c* to oxygen	0.0026	0.0866	16
GO:0006810	Transport	0.0034	0.0931	164
GO:0006979	Response to oxidative stress	0.0039	0.0931	43
GO:0042157	Lipoprotein metabolic process	0.0040	0.0931	16

In this case, the *P*- and *Q*-values relate to the significance of the difference in rank positions against that of all gene probes.

activity of the LC PUFA biosynthesis pathway was for instance increased in fish fed vegetable oils as a replacement for fish oil in the diet (Bell, McEvoy, Tocher, McGhee, Campbell & Sargent 2001). This might be due to a suppression in enzyme activity by higher n-3 LC PUFA levels (Zheng, Tocher, Dickson, Bell & Teale 2004). It is possible that no increase in LC PUFA by sesamin was observed in the present study because our experimental diets contained quite a high level of n-3 LC PUFA itself and n-3 LC PUFA biosynthesis suppression could occur. (3) In our study, we used pure sesamin, which differs from the study by Trattner, Kamal-Eldin *et al.* (2008), where an equimixture of sesamin/episesamin (1:1 w/w) was used. Kushiro, Masaoka, Hageshita, Takahashi, Ide and Sugano (2002) studied the modulatory effects of sesamin and episesamin on the lipid metabolism in rat liver and found that episesamin was more effective in increasing enzymatic activity. Therefore, episesamin rather than sesamin may be primarily responsible for the observed effects in rainbow trout *in vivo* and in cultivated Atlantic salmon hepatocytes (Trattner, Kamal-Eldin *et al.* 2008; Trattner, Ruyter *et al.* 2008). Detailed studies are required to investigate whether sesamin has the same effect as episesamin or whether they have synergistic effects in fish. (4) There are also other aspects that remain to be investigated, e.g. whether the sesamin effects, if any, on LC PUFA biosynthesis are age and size dependent. Zheng *et al.* (2004) reported that LC PUFA biosynthesis in Atlantic salmon varies during the growth cycle, with a higher activity in the young stage, then with peak activity occurring around seawater transfer and with a decrease in the older stages. Potential differences in the rate of LC PUFA biosynthesis between juveniles and adults might subsequently result in various effects of sesamin.

In the present study, carp white muscle lipids contained twofold higher levels of sesamin compared with rainbow trout (Trattner, Kamal-Eldin *et al.* 2008), which may have been caused by the longer experimental duration and therefore longer fish exposure to the dietary sesamin in the present study.

Higher EROD activity and a higher content of total CYP P450 were also observed in the sesamin-supplemented group, as reported in rainbow trout liver (Trattner, Kamal-Eldin *et al.* 2008). This indicates that carp also recognize sesamin as a xenobiotic compound. However, this is in disagreement with Parker, Sontag and Swanson (2000), who observed inhibition of the cytochrome system by sesamin in rat hepatocytes.

We have profiled the global gene expression properties with a homologous, high-density cDNA microarray to explore system-wide responses to sesamin treatment. Other experiments have demonstrated the power of this technique to provide a sensitive measure of responses across large numbers of genes from which definable patterns of response can be discerned (Gracey, Fraser, Li, Fang, Brass, Rogers & Cossins 2004). This includes the responses of lipid metabolism genes such as Δ9 and Δ6 acyl CoA desaturases, FA-binding proteins, etc. We have used a more powerful, dye-balanced ANOVA design with sixfold biological replication to provide extra statistical power to the analysis. Yet, while sesamin treatment was linked to an altered expression of 662 genes, we were unable to define any viable pattern to the responding genes that might signal changes in intermediary and particularly lipid metabolism.

In conclusion, under the conditions of our trial, we have not seen that sesamin alters LC PUFA biosynthesis in common carp. A possible explanation could be the high level of DHA in the diets, leading to a

decreased requirement of DHA synthesis. Therefore, we suggest that the metabolism of essential FA and LC PUFA in common carp should be investigated more systematically.

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Chapter 4

Lipid content and composition in common carp – optimization of n-3 fatty acids in different pond production systems

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Lipid content and composition in common carp – optimization of n-3 fatty acids in different pond production systems

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Summary

This study examined whether the lipid composition of common carp muscle can be improved and become an important source of n-3 highly unsaturated fatty acids (HUFA) in human nutrition. Carp from three pond production systems in South Bohemia were given access to plankton, or plankton with addition of cereals or pellets containing rapeseed cake. White muscle fatty acid composition was investigated and compared between treatments. The fish without any supplementation were characterized by a high content of n-3 HUFA, especially eicosapentaenoic (EPA) and docosahexaenoic acid (DHA). The fish supplemented with rapeseed pellets had a moderate n-3 HUFA level, while those supplemented with cereals were characterized by a high content of oleic acid and low level of n-3. The pond plankton populations contained high proportions of n-3 fatty acids, including EPA and DHA with an n-3/n-6 ratio around 3.

Keywords: Fatty acids, *Cyprinus carpio*, pond, plankton, flesh quality

1. Introduction

Fish are the most important food source of n-3 highly unsaturated fatty acids (carbon chain length ≥ C20 with ≥ 3 double bonds (n-3 HUFA); Sargent et al., 1989), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the human diet. The importance of n-3 fatty acids, especially n-3 HUFA for human health, has been reviewed in a number of publications (Calder, 2008; Mozaffarian et al., 2006; Okuyama et al., 1996; Simopoulos, 1991; 2008; Vrablík et al., 2009) and there is a large amount of evidence on the positive health effects of fish consumption. Fish intake is generally low in the Czech population (5.5 kg of fish products per capita in 2008; (MZe, 2009)) and it is far below current recommendations (EFSA, 2009; Kris-Etherton et al., 2002). Locally produced freshwater fish is not generally recognized as a good source of these fatty acids and their role in the food chain of man is underestimated (Ackman, 2002).

Common carp (*Cyprinus carpio*), which is one of the most important farmed freshwater fish species in central Europe, is a well-established cultured species with a well-known production cycle and is consumed as traditional food (Linhart et al., 2002). Carp is traditionally reared in earthen ponds and the diet is based on natural food in the ponds, with cereal supplementation. Since cereals are rich in carbohydrates and have very low levels of n-3 fatty acids, the flesh of farmed carp generally contains a high level of oleic acid and a low level of favourable n-3 HUFA (Csengeri, 1996).

The n-3 HUFA content in pond-produced carp flesh can be increased by several means, of which nutrition is the most important (Steffens, 1997). Carp is an omnivore and its natural

food consists mainly of zooplankton, zoobenthos and detritus (Adamek et al., 2004; Adamek et al., 2003). Plankton (Domaizon et al., 2000; Linko et al., 1992) and benthos (Bell et al., 1994; Bogut et al., 2007) naturally contain high levels of n-3 FA, including EPA and DHA. Thus proper pond management maintaining sufficient amounts and appropriate structure of planktonic and benthic communities is highly important when improving carp fatty acid composition.

Carp have the ability to biosynthesise n-3 HUFA from its precursor 18:3n-3, alpha-linolenic acid (ALA) (Tocher, 2003). Thus a feed supplement that is rich in ALA could be an alternative way to increase n-3 HUFA content in carp flesh. Feeds with a high level of ALA that are cheap and easily available include rapeseed, linseed and hempseed. Rapeseed or rapeseed cake is becoming an important component of pellets for carp nutrition in the Czech Republic owing to its low price and good availability. Rapeseed oil has a moderate amount of ALA (13%) and a favourable ratio n-6/n-3 of around 2 (Pickova et al., 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 the impact of rapeseed oil on carp lipid content and composition when used in pond production systems. In such systems there are many factors influencing muscle lipid composition, such as variations in amount and composition of natural food, fish density, environmental conditions and interactions between these.

The first part of the present study examined common carp flesh lipid quality in an extensive system with low carp density and without feed supplementation. The second part compared two semi-intensive systems with higher carp densities based on either cereal supplementation or supplementary pellets containing rapeseed cake.

2. Materials and methods

Experimental diets

The supplements used in the diet were wheat and the commercial feed pellets KP rapeseed (Výroba krmiv spol. s r.o., Czech Republic) containing rapeseed cake (30% of wet weight). The proximate composition of the diets (Table 1) was analyzed by the National Veterinary Institute in Prague (SVÚ Praha) according to standard methods for: ash, SOP nr. 26; dry matter, SOP nr. 25a; lipid, SOP nr. 21c; fibre, ČSN EN ISO 6865; protein, SOP nr. 23 (Kjeldahl); and nitrogen-free extract, ČSN 46 7092-24 (calculated from other analyses). The fatty acid composition of the diets (Table 1) was analyzed as already described by Mraz et al. (2009).

Experimental design

The experiment was performed in nine experimental ponds (all of the same size, 0.16 ha, with water taken from the Blanice River) at the Research Institute of Fish Culture and Hydrobiology in Vodňany. The study lasted one growing (vegetation) season (22/04/2008 – 15/10/2008). The Institute is located in South Bohemia, Czech Republic, Europe (temperate zone; approx. co-ordinates 49° 08' 52" N and 14° 10' 32" E; altitude 400 m above sea level; mean annual temperature 7.5 ºC and mean annual rainfall 550–650 mm). For the experiment, two-year-old common carp (crossbred Tata carp x Ropsha carp) with mean live weight 345.5 ± 115.1 g were used (33 individuals per pond were weighed individually for mean weight and variation). The carp were reared in three production systems (each in triplicate):

1) Extensive system ‘control’: low carp density (50/pond) no supplementary feed.
2) Semi-intensive system ‘wheat’: higher carp density (200/pond) wheat supplementation (520 kg wheat/pond).

3) Semi-intensive system ‘rapeseed’: higher carp density (200/pond) supplementary pellets containing rapeseed cake (442 kg pellets/pond). Since the expected feed conversion ratio of this product as stated by the manufacturer was better (2.1) than for wheat (2.5), the feed ratio was adjusted to 85% of that in the wheat group in order to obtain approximately the same individual live weight gain (1 kg) per individual.

The feed was supplied to the ponds five times per week in the morning (in feed ration equivalent to 1.5–3% of actual biomass adjusted to temperature, oxygen level and feed uptake). Doses of feed used in May, June, July, August and September comprised 4.5, 16, 25.5, 31 and 23% of the total feed. At the end of the experiment, fish were harvested, individually weighed and sampled (6 individuals/pond) for lipid analyses (~ 10 g of white dorsal muscle) after one day of fasting. Samples were frozen immediately in liquid nitrogen and stored at -80 °C prior to further analyses.

Water quality and plankton sampling

Zooplankton samples were taken on three occasions (2 June, 11 July 1 and September) from all the experimental ponds. These samples were collected with a plankton net (mesh size 80 μm) and stored for analysis of species (preserved with formaldehyde) and lipid composition (kept at -80 °C). During the experiment, the water temperature, dissolved oxygen content, pH (WTW2D30-100 pH-oximetr, WTW, s.r.o., Prague, Czech Republic) and transparency were measured twice a week in the morning (8.00–10.00 a.m.).

Lipid analyses

Lipid analyses were performed as already described by (Mraz et al., 2009). Lipids from tissues, diets and plankton were extracted by hexane-isopropanol (Hara et al., 1978). Total lipids were fractionated by thin layer chromatography for separation of lipid classes. Fatty acids were methylated and analyzed with a Varian CP3800 gas chromatograph (Stockholm, Sweden) equipped with flame ionization detector and 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). Fatty acids were identified by comparison with the standard mixture GLC-461 (Nu-check Prep, city, state) using retention time. Peak areas were integrated by means of Star chromatography workstation software version 5.5 (Varian AB, Stockholm, Sweden).

Statistics

The data were processed using the data analysis software STATISTICA CZ, v. 8, StatSoft, Inc. All values are expressed as mean ± standard deviation. For statistical analyses one-way ANOVA and Tukey’s post-hoc test with statistical level of significance $\alpha = 0.05$ were used.

3. Results

Production data

All the experimental groups showed a high survival rate, which ranged from 97.5 to 100%. The control group reared on the natural food had the highest final weight (1.89 ± 0.33 kg) and individual weight gain (1.54 ± 0.25). Intermediate final weight (1.48 ± 0.02) and individual weight gain (1.13 ± 0.04) were shown in the group receiving the wheat supplement. The group with the rapeseed pellet supplement had the lowest final weight (1.36 ± 0.05) and individual weight gain (1.01 ± 0.02) ($p < 0.05$). The total weight gain per pond was highest

for wheat, intermediate for rapeseed and lowest in the control group ($p < 0.05$). Feed conversion ratio did not statistically differ between wheat (2.35) and rapeseed groups (2.26) ($p > 0.05$) (Table 2).

White muscle lipid content and fatty acid composition

The white muscle lipid content was clearly affected by the pond production system. The highest lipid content was found in white dorsal muscle of wheat group carp (2.0 ± 0.3%), which was two-fold higher than in the control (1.0 ± 0.6%) and rapeseed group (0.9 ± 0.4%) ($p < 0.05$).

The white muscle fatty acid composition of all three lipid classes studied (total lipid, triacylglycerols (TAG) and phospholipids (PL)) was affected by the pond production system (Table 3). The PL class in all groups was characterized by a high level of HUFA and low level of other fatty acids, whereas TAG showed the opposite pattern. Saturated fatty acids (SFA) did not differ significantly between the carp groups within any lipid class. Percentage of monounsaturated fatty acids (MUFA) was lowest in the control group, moderate in the rapeseed group and highest in the wheat group. The rapeseed group had almost two-fold higher linoleic acid (LA) (18:2n-6) level compared with other groups within all lipid classes. The proportion of ALA and arachidonic acid (AA) (20:4n-6) was significantly higher in the control group compared with the others except for PL, where all groups had the same level of AA. EPA (20:5n-3) level was highest in the control group, moderate in the rapeseed group and the lowest in the wheat group within all lipid classes. DHA (22:6n-3) showed a similar pattern, but a significant difference was only seen in total lipid. The n-3/n-6 ratio was significantly higher in the control group in all lipid classes. In spite of the higher levels of n-3 HUFA in the rapeseed group compared with the wheat group, the ratio did not differ significantly.

Plankton fatty acid composition

The content of SFA, MUFA and polyunsaturated fatty acids (PUFA) ranged from 27.9–32.9%, 22.9–29.4% and 37.8–46.5% of total identified fatty acids, respectively (Table 4). All plankton samples were rich in n-3 HUFA, with a very favourable n-3/n-6 ratio of around 3. There were no statistical significant differences between the plankton from rapeseed group ponds and wheat group ponds within each sampling occasion. However, there were pronounced differences between the plankton from control group ponds and the other ponds within each sampling occasion. The plankton from the control group ponds had two-fold higher levels of AA and EPA compared with other ponds in samples taken on the first sampling occasion (2 June). On 11 July, the plankton from control group ponds had a two-fold higher level of ALA and a lower level of AA than other ponds, while on 1 September they had a higher level of MUFA, ALA and a very low level of DHA compared with other ponds. Seasonal variations in plankton FA composition occurred in all the ponds. However, pronounced changes were observed only in the plankton from the rapeseed and wheat group ponds, where the percentage of ALA decreased, whereas HUFA increased strongly during the period June–September. The plankton FA composition of the control group was more stable during the experiment and there were no pronounced differences between the sampling occasions except for minor changes in AA and MUFA.

Water quality and plankton community structure

Some of the water quality parameters differed substantially between control group ponds and the others, while the wheat and rapeseed group ponds did not differ significantly from each other. The substantial differences were mainly in transparency, which varied from 40 to 120 cm in the control ponds but was only 20 to 40 cm in the other ponds. The content of dissolved O_2 was highly variable, ranging from 0.8 to 10.8 O_2 mg L^{-1}. The pH varied in the range 7.34–9.73 (control) and 7.05–8.30 (wheat and rapeseed). Temperature ranged from 15 to 25 °C and did not differ between the ponds.

Plankton community structure varied between the ponds. A general pattern emerged in that the zooplankton biomass was dominated by cladocera early in the experiment but there was then a shift towards the smaller zooplankton species, copepoda and rotifera, except in the control group, where the community structure was relatively stable.

4. Discussion

Fish production

Supplementary feed increased carp yield three-fold compared with the control group. We expected the rapeseed group to have better conversion ratio and the same weight gain with the adjusted feeding ration (85% of the wheat) as the wheat group, but this was not the case. The conversion ratio of the rapeseed group was indeed slightly better than for wheat but the difference was not as high as expected (stated feed conversion ratio 2.1 for rapeseed pellets and 2.5 for wheat). Consequently, the weight gain of rapeseed-supplemented fish groups was slightly lower than for wheat groups. Nevertheless, apart from the control group, the final weight of both groups was approximately the same, which was the aim, so that we could compare the lipid content and composition of the fish. No differences were observed in the feed supplements in terms of rejection by fish and all feeds were eaten very rapidly.

Effects on carp muscle lipids

The FA composition of carp from the control group reflected the FA composition of plankton to a very great extent. However, the level of DHA in the carp muscle was almost four-fold higher than in plankton. Different metabolic pathways probably caused this specific retention of DHA. Since the plankton FA composition in control ponds was relatively stable across all sampling occasions, the results may indicate a high bioconversion rate to DHA in carp. Other possible explanations are selective storage, retention of DHA or feed selectivity.

Supplementary wheat doubled lipid content and increased MUFA, whereas rapeseed caused higher levels of PUFA and HUFA. It is known that MUFA are either produced by desaturation of saturated FA synthesized in the organism from energy-rich feed (Csengeri, 1996; Henderson, 1996), in the way that other vertebrates desaturate this compound by Δ-9 desaturase, or stored directly in adipocytes.

Impact of farming on water quality, plankton lipids and community structure

Water quality and plankton community structure and its lipid composition were influenced by rearing system. Differences were found only between control group ponds and both semi-intensive systems. These changes probably arose mainly due to differing carp density and nutrient level. Water transparency was significantly lower in the ponds with nutrient supplementation, with both wheat and rapeseed pellets having the same effect, possibly

reflecting the nutrient level. High fish stocking density causes high predation pressure on zooplankton and suppresses large species of *Daphnia* in particular (Ingram, 2009). Consequently, phytoplankton are not effectively controlled by small zooplankton species and the efficiency and grazing of the high primary production is low, as suggested by (Potužák et al., 2007). As a result of this effect, a shift in plankton community structure from cladocera towards copepoda was seen. The observed changes in plankton fatty acid composition throughout the experiment from EPA being the predominant n-3 HUFA to DHA predominating are in agreement with (Persson et al., 2006), who reported EPA to be typical of cladocera, whereas DHA are more frequent in copepoda.

Importance of carp muscle fatty acid composition in human nutrition

Many leading authorities and nutrition and health organizations have developed specific dietary recommendations for omega-3 fatty acids and fish intake for different countries around the world. The UK government recommendation is to eat at least two portions of fish a week, one of which should be oil-rich (SACN/COT, 2004). The American Heart Association recommends eating fish (particularly fatty fish) at least two times a week (Kris-Etherton et al., 2002). The European Food Safety Authority recommends 2 g of n-3 PUFA per day and 250 mg of EPA and DHA per day for the general adult population (EFSA, 2009). Mozaffarian et al. (2006) recommend 250 mg of EPA and DHA per day for the general population to reduce mortality from CHD (cardiac and heart disease). However, Okuyama et al. (1996) recommend an n-3/n-6 PUFA ratio of 1:1-2 for prevention of certain chronic diseases instead of the amount of individual FA. Simopoulos (2008) suggested that the n-3/n-6 ratio should be kept between 1:1-4. It has been estimated that the Western diet is deficient in omega 3 fatty acids with an n-3/n-6 ratio of 1:15-20 (Eaton et al., 1985; Simopoulos, 1991).

Actual intake of fish products in the Czech population is far below the target level. To meet the recommendations, it would be necessary to increase consumption from the current level of around 5.5 to 20 kg of fish per capita and year.

In the present study, we analyzed white dorsal muscle, which is the leanest part of carp fillet (Mraz et al., 2009). A 200 g serving of white muscle from control, rapeseed and wheat group carp contained 173 mg, 90 mg and 87 mg of EPA+DHA, respectively (whole fillet 300 mg, 170 mg and 150 mg, respectively) and 344 mg, 127 mg and 125 mg of n-3 PUFA, respectively. The n-3/n-6 ratio in white muscle of control, rapeseed and wheat group carps was 1:0.54, 1:1.53 and 1:2.22, respectively. There is a great potential to improve feeding strategies in carp production to achieve higher fatty acid production and a better HUFA profile. Our findings of 290 mg EPA+DHA and 880 mg of n-3 PUFA per 200 g serving from rapeseed/linseed mixture pellets (unpublished results, n = 5) are promising.

Based on the data presented here, carp from all three production systems studied had favourable FA composition and should be regarded as a healthy product. To further improve fatty acid composition in the rapeseed group in order to reach the recommended values, we suggest replacing approximately half the rapeseed cake in pellets with linseed. This should result in higher n-3 PUFA levels and could lead to increased DHA levels due to conversion of ALA. The conclusion is that rapeseed cake can be used as a valuable component of a supplemented carp diet to improve muscle fatty acid composition.

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Table 1. Proximate composition (g/100g) and fatty acid composition (%; mean ± SD; n=4/ group) of the experimental diets. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids.

	KP rapeseed	**Wheat**

Dry matter	88.8	88.3
Protein	15.7	10.4
Lipids	6.9	2.4
Ash	5.5	1.7
Fibre	7.3	1.8
Nitrogen-free extract	53.3	72.0
SFA	13.6 ± 0.6	18.3 ± 0.1
MUFA	37.9 ± 3.4	16.3 ± 0.2
18:2 (n-6)	42.9 ± 3.7	61.3 ± 01
18:3 (n-3)	5.6 ± 1.0	4.2 ± 0.1
Σ n-3/Σ n-6	0.13 ± 0.03	0.07 ± 0.0

Table 2. Production data on carp reared in different production systems. Data are presented as mean ± SD. Different letters within rows show statistically significant difference among groups ($P<0.05$, $n=3$).

	Control	**Wheat**	**Rapeseed**
Number of fish	50	200	200
Initial total weight (kg)	19.8 ± 1.69 a	69.1 ± 5.31 b	69.1 ± 5.31 b
Initial individual weight (kg)	0.346 ± 0.11 a	0.346 ± 0.12 a	0.346 ± 0.12 a
Survival (%)	100 ± 0.0 a	98.3 ± 1.3 a,b	97.5 ± 0.7 b
Final total weight (kg)	94.5 ± 11.1 a	290.9 ± 3.23 b	265.2 ± 8.69 c
Final individual weight (kg)	1.89 ± 0.33 a	1.48 ± 0.02 b	1.36 ± 0.05 c
Total weight gain (kg)	77.2 ± 12.6 a	221.8 ± 8.5 b	195.9 ± 3.4 c
Individual weight gain (kg)	1.54 ± 0.25 a	1.13 ± 0.04 b	1.01 ± 0.02 c
Consumed feed (kg)	–	520	442
Feed conversion ratio	–	2.35 ± 0.09 a	2.26 ± 0.04 a

Table 3. White muscle fatty acid composition (% of identified fatty acids) of carp from different production systems (mean ± SD; n = 9 for each group); SFA = saturated fatty acids; MUFA = monounsaturated fatty acids. Different letters within row shows statistically significant difference ($P < 0.05$) between groups within the same lipid class. The factor used for weight calculations of total lipid composition (mg/g of lipid) for control, rapeseed and wheat was 6.13, 6.63 and 8.18, respectively.

	Total lipid			**Triacylglycerols**			**Phospholipids**		
	Control	**Rapeseed**	**Wheat**	**Control**	**Rapeseed**	**Wheat**	**Control**	**Rapeseed**	**Wheat**
SFA	28.2 ± 1.63	28.0 ± 1.24	27.4 ± 1.00	29.1 ± 3.53	27.4 ± 1.15	28.2 ± 1.80	29.2 ± 0.67	30.1 ± 4.60	28.0 ± 2.17
MUFA	29.0 ± 8.17a	39.8 ± 7.15b	54.4 ± 6.50c	44.1 ± 5.38a	55.3 ± 2.20b	62.0 ± 2.56c	16.6 ± 0.93a	22.9 ± 4.23b	28.0 ± 1.28c
18:2n-6	5.59 ± 0.77a	10.0 ± 1.32b	6.32 ± 0.96a	8.05 ± 1.24a	12.2 ± 1.34b	6.57 ± 1.01a	3.93 ± 1.55a	6.61 ± 1.11b	4.79 ± 0.44a
18:3n-6	0.20 ± 0.13	0.18 ± 0.07	0.17 ± 0.02	0.31 ± 0.18a	0.21 ± 0.04ab	0.16 ± 0.02b	0.06 ± 0.05	0.09 ± 0.06	0.07 ± 0.08
18:3n-3	6.13 ± 3.95a	1.47 ± 0.35b	0.67 ± 0.09b	8.91 ± 5.14a	1.85 ± 0.28b	0.73 ± 0.18b	2.92 ± 1.21a	0.78 ± 0.06b	0.44 ± 0.10b
18:4n-3	–	–	–	0.68 ± 0.71	0.47 ± 0.81	0.59 ± 0.84	0.20 ± 0.11	0.22 ± 0.53	0.20 ± 0.10
20:2n-6	0.49 ± 0.14a	0.57 ± 0.11a	0.35 ± 0.07b	0.46 ± 0.09a	0.42 ± 0.06a	0.28 ± 0.07b	0.59 ± 0.18	0.76 ± 0.17	0.60 ± 0.14
20:3n-6	0.60 ± 0.13a	1.06 ± 0.28b	0.63 ± 0.24a	0.28 ± 0.14	0.32 ± 0.02	0.24 ± 0.04	0.77 ± 0.16a	1.57 ± 0.27b	1.60 ± 0.20b
20:4n-6	6.49 ± 2.49a	5.13 ± 1.85ab	2.83 ± 1.57b	1.47 ± 0.50a	0.70 ± 0.09b	0.59 ± 0.10b	10.2 ± 0.54	10.1 ± 1.86	10.7 ± 1.27
20:3n-3	0.53 ± 0.19	–	–	0.38 ± 0.34a	0.07 ± 0.05b	0.01 ± 0.02b	0.66 ± 0.22a	0.12 ± 0.12b	0.04 ± 0.06b
20:4n-3	0.82 ± 0.35a	0.24 ± 0.11b	0.12 ± 0.13b	0.53 ± 0.46a	0.12 ± 0.09b	0.04 ± 0.06b	1.05 ± 0.50a	0.34 ± 0.15b	0.23 ± 0.22b
20:5n-3	7.57 ± 1.92a	2.28 ± 0.85b	0.94 ± 0.61c	3.53 ± 1.60a	0.48 ± 0.08b	0.27 ± 0.07c	9.65 ± 1.45a	3.85 ± 0.82b	2.67 ± 0.45c
22:4n-6	0.68 ± 0.28a	0.52 ± 0.19ab	0.32 ± 0.16b	0.20 ± 0.13a	0.03 ± 0.04b	0.05 ± 0.04b	1.18 ± 0.18	1.07 ± 0.24	1.15 ± 0.15
22:5n-6	0.93 ± 0.62	1.22 ± 0.47	0.71 ± 0.44	–	–	–	1.67 ± 0.34a	2.29 ± 1.03ab	3.13 ± 0.57b
22:5n-3	3.65 ± 1.54a	1.56 ± 0.60b	0,73 ± 0.52b	1.02 ± 0.39a	0.15 ± 0.07b	0.12 ± 0.02b	5.69 ± 0.60a	3.11 ± 0.60b	2.57 ± 0.32b
22:6n-3	8.37 ± 4.40a	6.76 ± 2.96b	2.95 ± 2.21c	1.00 ± 0.32a	0.31 ± 0.13b	0.18 ± 0.04b	15.2 ± 2.37	14.6 ± 3.09	12.5 ± 1.15
n-3/n-6	1.84 ± 0.23a	0.65 ± 0.18b	0.45 ± 0.21b	1.49 ± 0.63a	0.25 ± 0.07b	0.25 ± 0.13b	1.94 ± 0.29a	1.03 ± 0.15b	0.86 ± 0.16b

Table 4. Plankton fatty acid composition (% of identified fatty acids) in different production systems on three sampling occasions (mean ± SD; n = 9 for each group) SFA = saturated fatty acids; MUFA = mono unsaturated fatty acids. Different letters x, y, z show statistically significant difference (P < 0.05) between sampling occasions within the same group.

	02/06/2008			11/07/2008			01/9/2008		
	Control	**Rapeseed**	**Wheat**	**Control**	**Rapeseed**	**Wheat**	**Control**	**Rapeseed**	**Wheat**
SFA	28.2 ± 0.3	32.8 ± 1.5x	29.7 ± 3.0	31.8 ± 3.1	28.6 ± 1.1y	31.0 ± 3.6	27.9 ± 3.6	30.6 ± 2.2xy	32.9 ± 1.4
MUFA	27.8 ± 2.3xy	29.4 ±3.5	27.2 ± 5.4	24.4 ±1.6x	29.1 ± 3.0	25.3 ± 3.9	29.2 ± 0.3ay	22.9 ± 3.2b	23.6 ± 2.5ab
18:2n-6	6.0 ± 0.3	6.6 ± 0.5	7.1 ± 1.5	5.3 ± 0.8	6.5 ± 0.6	5.8 ± 0.5	4.3 ± 1.2	6.3 ± 0.3	5.6 ± 0.8
18:3n-6	0.8 ± 0.1	0.7 ± 0.1xy	0.8 ± 0.1	0.7 ± 0.1	0.9 ± 0.1x	0.9 ± 0.3	0.7 ± 0.2	0.6 ± 0.1y	0.8 ± 0.1
18:3n-3	13.8 ± 2.3	17.4 ± 1.9x	16.9 ± 3.9x	19.5 ± 3.6a	10.8 ± 0.3by	11.2 ± 0.8bxy	16.6 ± 3.9a	9.9 ± 1.3by	8.7 ± 0.9by
20:2n-6	0.1 ± 0.2	0.1 ± 0.1x	0.1 ± 0.2	0.3 ± 0.1	0.2 ± 0.1xy	0.3 ± 0.1	0.2 ± 0.2	0.4 ± 0.2y	0.3 ± 0.1
20:3n-6	0.3 ± 0.1	0.2 ± 0.2x	0.4 ± 0.2x	0.3 ± 0.1	0.4 ± 0.0xy	0.5 ± 0.1xy	0.3 ± 0.2	0.4 ± 0.1y	0.7 ± 0.4y
20:4n-6	5.8 ± 1.0ax	2.5 ± 0.5bx	3.0 ± 0.7bx	2.6 ± 0.7ay	4.0 ± 0.6abxy	4.9 ± 0.8by	3.9 ± 1.8xy	4.4 ± 0.7y	5.4 ± 0.6y
20:3n-3	0.2 ± 0.2	0.1 ± 0.2x	0.2 ± 0.3	0.4 ± 0.2	0.4 ± 0.1xy	0.5 ± 0.2	0.3 ± 0.1	0.6 ± 0.1y	0.5 ± 0.1
20:4n-3	0.5 ± 0.1	0.6 ± 0.2x	0.7 ± 0.2	1.0 ± 0.2	0.7 ± 0.1xy	1.0 ± 0.3	1.1 ± 0.5	1.0 ± 0.1y	1.1 ± 0.6
20:5n-3	14.3 ± 2.9a	7.4 ± 1.1bx	9.2 ± 1.7b	9.7 ± 1.1	11.3 ± 0.4y	10.9 ± 1.5	12.7 ± 0.9	10.3 ± 1.7xy	11.7 ± 0.1
22:5n-3	0.3 ± 0.1	0.3 ± 0.1x	0.3 ± 0.5	0.3 ± 0.1	0.5 ± 0.2xy	0.7 ± 0.3	0.3 ± 0.1	0.9 ± 0.2y	0.9 ± 0.4
22:6n-3	1.8 ± 0.8	1.9 ± 1.2x	4.4 ± 5.8	3.7 ± 1.3	6.7 ± 3.1xy	7.1 ± 3.0	2.5 ± 1.0a	11.5 ± 2.8by	7.8 ± 1.6b
n-3/n-6	2.4 ± 0.3	2.7 ± 0.3	2.8 ± 0.7	3.8 ± 0.7	2.6 ± 0.5	2.6 ± 0.6	3.9 ± 1.8	2.9 ± 0.5	2.4 ± 0.2

Chapter 5

Effects of bioactive compounds on fatty acids and gene expression involved in hepatocyte lipid metabolism of Atlantic salmon (*Salmo salar* L.)

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Effects of bioactive compounds on fatty acids and gene expression involved in hepatocyte lipid metabolism of Atlantic salmon (*Salmo salar* L.)

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Abstract

Introduction

Due to shortage of fish oil and expected increase in aquaculture production, research is focusing on replacement of oils in aqua feeds with increasing amounts of vegetable oils (Tacon et al., 2008). It is well known that replacement of fish oil with vegetable oils decreases the content of n-3 long chain polyunsaturated fatty acids (LCPUFA) in the fish tissues (Torstensen et al., 2005). This decrease is expected to be negative for consumer health as LCPUFA are beneficial for protection from cardiac diseases and others (Mozaffarian et al., 2006). In terms of the consumer's health, it would be beneficial to maintain as high as possible amounts of n-3 LCPUFA in fish muscle (Ackman, 1996).

A number of bioactive substances, e.g., sesamin, episesamin, lipoic acid and tetradecylthioacetic acid (TTA) has been reported to affect lipid metabolism and/or fatty acid (FA) composition in pacu (*Piaractus mesopotamicus*), rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar* L) (Moya-Falcón et al., 2004; Trattner et al., 2007; Trattner et al., 2008a; Trattner et al., 2008b). Bioactive compounds are naturally occurring components in or derived from plant products and foods rich on lipids. They are present in small amounts and evoke health beneficial effects beyond the products basic nutritional value (Kitts, 1994). It has been suggested that bioactive compounds play very different roles spanning from being antioxidants, having chemopreventive abilities in certain cancer forms to lowering blood pressure and decreasing the incidence of cardiovascular disease, coronary heart disease and stroke (Kris-Etherton et al., 2002). Many bioactive compounds have been shown to markedly influence lipid homeostasis in rodents (Huong et al., 2008; Kushiro et al., 2002; Lim et al., 2007) as well as in humans (Crouse Iii et al., 1999; Jenkins et al., 2000; Miettinen et al., 2003). From the perspective where bioactive compounds act as antioxidants or directly influence lipid homeostasis, they are of interest as potential constituents in fish feed possibly promoting the ability of fish to convert α-linolenic acid (18:3n-3, ALA) into eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA).

Sesamin is an oil soluble lignan abundantly found in seed and oil of the sesame plant (Ide et al., 2003). Episesamin is not a naturally occurring compound but is formed from sesamin

during the refining process of the sesame oil. Therefore, commercially available sesamin contain approximately equal amounts of sesamin and episesamin (Fukuda et al., 1986). The effect of these sesame lignans on lipid metabolism are well documented in mammals. Sesamin/episesamin has been shown to increase β-oxidation in rodents through activation of peroxisome proliferator-activated receptor α (PPARα) (Ashakumary et al., 1999), affect elongation and desaturation of FAs in rats (Fujiyama-Fujiwara et al., 1995) as well as lowering serum levels of triacylglycerols and cholesterol in rats and humans (Kamal-Eldin et al., 2000; Kushiro et al., 2002). Sesamin/episesamin has been shown to increase the levels of docosahexaenoic acid (DHA, 22:6n-3) in rainbow trout muscle (Trattner et al., 2008b) as well increase β-oxidation products and expression of Δ5 and Δ6 desaturase, carnitine palmitoyltransferase 1 (CPT1), peroxisome proliferators-activated receptor α (PPARα) and PPARγ in Atlantic salmon hepatocytes (Trattner et al., 2008a).

Lipoic acid is a potent antioxidant with one lipophilic and one lipofobic part, having vitamin C and vitamin E sparing effects in mammals (Kozlov et al., 1999). Lipoic acid has been shown to increase the portion of eicosapentaenoic acid (EPA, 20:5n-3) levels in pacu muscle (Trattner et al., 2007).

Genistein is isoflavone formed after hydrolysis of the β-glycoside genistin found in soy with antioxidative and hormone like effects (Messina et al., 2006). It has been found to inhibit the oxidation of low-density lipoprotein (LDL) in human blood (Safari et al., 2003) and decrease the serum total cholesterol (Zhan et al., 2005). According to Mezei (2006) soy isoflavone intake alter lipid metabolism in a manner consistent with activation of PPARα and also via a PPARα-independent mechanism as well.

Atlantic salmon hepatocytes are an established in vitro system and have been used in a range of metabolic studies (Kjær et al., 2008; Krøvel et al., 2010; Torstensen et al., 2004; Trattner et al., 2008a; Zhou et al., 2010) offering a unique opportunity to study the effect of bioactive compounds on lipid metabolism.

This study aims to investigate the effects of different selected bioactive compounds- the mixture of sesamin/episesamin, sesamin, lipoic acid and genistein on FA profile and the gene expression profile associated with lipid homeostasis in hepatocytes isolated from Atlantic salmon fed with standard fish oil diet. Our anticipation is that understanding how bioactive compounds effect the regulation of the molecular mechanisms for FA uptake, LCPUFA biosynthesis, mitochondrial and peroxisomal β-oxidation, we may identify tools for optimization of the activity of the sequential desaturation and elongation of PUFA. By adding bioactive compound to the fish feed we hope to enable an efficient use of vegetable oils without a negative impact on the nutritional value of fish.

Material and methods

Chemicals and reagents

The linolenic acid 18:3n-3, essential fatty acid free bovine serum albumin (BSA), collagenase type 1, dimethyl sulfoxide (DMSO), Phosphate buffer saline (PBS), phenylethylamine and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), sodium bicarbonate solution, genistein and lipoic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and methacain MS-222 from Norsk Medisinaldepot AS (Olso, Norway). Fetal bovine serum (FSB) and GlutaMAX™ were supplied from Invitogen by Life Technologies (Carlsbad, CA, USA) and Penicillin/Streptomycin ThermoFisher Scientific (Waltham, MA, USA), laminin from Millipore (Billerica, MA, USA). Sesamin (sesamin/episesamin mixture, 1:1, w/w) was a kind gift from Takemoto Oil and Fat Co., Ltd. (Gamagori Aichi, Japan) and sesamin was obtained from KEB Biotech (Beijing, China). FA

peaks were identified by comparison with the standard mixture GLC-68 A (Nu-check Prep, Inc, Elysian, Minnesota, USA). All solvents and other chemicals for FA analysis were purchase from Merck (Darmstadt, Germany).

Isolation of hepatocytes

Atlantic salmon of approximately 1300 g (NIVAs Research Station, Drøbak, Norway) was kept in seawater at 10 °C and fed a commercial diet prior to isolation of hepatocytes. The fish were anesthetized with methacain, killed, the abdorminal cavity exposed and the portal vein cannulated. The parenchymal cells were obtained and hepatocytes were isolated according to the two-step collagenase procedure developed by Seglen (1976) and modified by Dannevig and Berg (1985) and conducted as Kjær et al. (2008). After collagenase perfusion, the parenchymal cells were isolated by gently shaking the liver in GlutaMAX culture medium. The suspension of parenchymal cells was filtered through a 100-µm nylon filter and the hepatocytes were isolated from the filtrate by three times centrifugation, each time for 2 min at 50×g. The hepatocytes were resuspended in Leibowitz -15 culture medium containing 2% FBS, 2 mM L-glutamine, and 0.1 mg/mL gentamycin.

Incubation of hepatocytes

The isolated hepatocytes were incubated in 5 ml of GlutaMAX culture medium with FBS for 18hr (the fatty acid composition of GlutaMAX + FBS was: 14:0 = 8.4%; 14:1 = 0.6%; 15:0 = 2.3%; 16:0 = 31.7%; 16:1 = 4,1%; 18:0 = 31.7%; 18:1 n-9 = 14.9%; 18:1n-7 = 2.7%; 18:2n-6 = 3.7%; 18:3n-3 = 1.0%; 20:3 n-6 = 0.8%; 20:4n-6 = 2.4%; 20:5n-3 = 0.5%; 22:5n-3 = 0.9%; 22:6n-3 = 1.2%), then washed and incubated with 35 nmol 18:3n-3, final fatty acid concentration of 7µM, in 5 ml of GlutaMAX culture medium without FBS for at 12 ºC for 12 respectively 48 hr. The fatty acid was added to the medium as potassium salt bound to BSA. For each treatment 9 flasks were incubated, the treatments were: I) genistein 0.005mM final concentration, II) genistein 0.025mM final concentration, III) lipoic acid 0.2mM final concentration, IV) sesamin/episesamin 0.05mM final concentration, V) sesamin 0.05mM final concentration. To 9 controls 7.4µl DMSO/5 ml medium was added and to another set of controls nothing was added. The bioactive substances were added dissolved in DMSO and given in the same amount as the DMSO control. Sampling for gene expression analysis occurred at two different time points, after 12 and 48 hr incubation. The cells were washed in PBS prior to isolation of mRNA for gene expression analysis. The cells for FA analyses washed in PBS with 1% albumin and scraped off in PSB after 48 hr incubation. At each sampling point and for each analyse the number of samples were n=3.

Analysis of lipids in cells and medium

Lipids from the cells and the medium were extracted by using hexane:isopropanol (3:2 by vol.) (Hara et al., 1978). Total lipids were converted to FA methyl esters by using 20% boron trifluoride-methanol complex according to Appelqvist (1968) and analysed with Gas Chromatograph CP3800 (Agilent Technologies, Santa Clara, CA, USA) according to Fredriksson et al. (2007).

Total RNA extraction, cDNA synthesis and real time PCR

Total RNA was isolated from the cells using the RNeasy® Mini Kit with on-column Rnase free DNase set (Qiagen, MD, USA). All protocols were according to the manufacturer's

instructions. Purity and density were measured by optical density (NanoVue, Spectrophotometer, GE Healthcare Life Sciences, Uppsala, Sweden) and samples were stored in RNase-free water (Eppendorf, Hamburg, Germany) at -70 ºC. The cDNA was synthesized following the protocol from the ImProm-IITM Reverse Transcription System (Promega, MD, USA). A mixture of the Oligo d(T)15 and random primers were used. The reaction was performed by incubating the samples at 25 ºC for 5 min, 42 ºC for 60 min, 75 ºC for 15 min.

Table 1. Sequences of primers and genbank accession numbers used

Primer	Forward primer (5'-3')	Reverse primer (5'-3')	GenBank Acc. no
RPL2[a]	TAACGCCTGCCTCTTCACGTTGA	ATGAGGGACCTTGTAGCCAGCAA	CA049789
EF1-α[b]	CACCACCGGCCATCTGATCTACAA	TCAGCAGCCTCCTTCTCGAACTTC	AF321836
PPARα[a]	TCCTGGTGGCCTACGGATC	CGTTGAATTTCATGGCGAACT	DQ294237
PPARβ1A[a]	GAGACGGTCAGGGAGCTCAC	CCAGCAACCCGTCCTTGTT	AJ416953
PPARγ[a]	CATTGTCAGCCTGTCCAGAC	ATGTGACATTCCCACAAGCA	AJ292963
CD36[d]	GGATGAACTCCCTGCATGTGA	TGAGGCCAAAGTACTCGTCGA	AY606034
Δ5 [d]	GAGAGCTGGCACCGACAGAG	GAGCTGCATTTTTCCCATGG	AF478472
Δ6 [d]	AGAGCGTAGCTGACACAGCG	TCCTCGGTTCTCTCTGCTCC	AY458652
ACO[a]	CCTTCATTGTACCTCTCCGCA	CATTTCAACCTCATCAAAGCCAA	DQ364432
CPT1 [d]	GTACCAGCCCCGATGCCTTCAT	TCTCTGTGCGACCCTCTCGGAA	AM230810
elovl5a[c]	ACAAGACAGGAATCTCTTTCAGATTAA	TCTGGGGTTACTGTGCTATAGTGTAC	AY170327
elovl2[c]	CGGGTACAAAATGTGCTGGT	TCTGTTTGCCGATAGCCATT	TC91192

Abbreviations: RPL2 = RNA polymerase II polypeptide, EF1A = Elongation factor 1α, PPAR = peroxisome proliferator-activated receptor, CD 36 = cluster of differentiation 36, Δ5 = Δ5 desaturase, Δ6 = Δ6 desaturase, ACO = acyl-CoA oxidase, CPT1 = carnitin palmitoyl transferase I. Already designed and validated in a)(Kleveland et al., 2006) b)(Jorgensen et al., 2006) c)(Morais et al., 2009) d)(Trattner et al., 2008a)

Primers for Real-Time PCR analysis (Table 1) had previously been designed based on available salmon or rainbow trout sequences in the GenBank® and ordered from Invitrogen (Carlsbad, CA, USA). Real-Time PCR was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with gene-specific primers for PPARα, β, γ and cluster of differentiation 36 (cd36), Δ5 desaturase, Δ6 desaturase, acyl-CoA oxidase (ACO) and carnitin palmitoyl transferase I (CPT1). A 2xPower SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used in the PCR reaction mix of 20 µl with 4 µl primers (final concentration of 0.5 µM), and 10 µl cDNA. All samples were analyzed in triplicate with non-template control on each plate. The reaction was incubated at 95 °C for 5 min, 45 cycles of 95 °C for 10s, 60 °C for 15s and 72 °C for 15s. The ΔCT was calculated and the reference genes were evaluated using the DataAssist software version 2.0 (Applied Biosystems, Foster City, CA, USA). RPL2 was chosen as reference gene, having the best stability (0.9586) over all samples and treatments. The relative expression was then calculated comparing the ΔCT values for hepatocytes with or without addition of additive using the term $2^{-\Delta\Delta CT}$ and reported as arbitrary fold change units (Livak et al., 2001).

Statistical analysis

Relative expressions of the different genes, in relation to the reference gene, were determined using the DataAssist software version 2.0 (Applied Biosystems, Foster City, CA, USA). For all other data, the student t-test in Microsoft Office Excel 2003 was used.

Results

Fatty acid composition

After 48 h of incubation, the proportion of 18:4n-3 was significantly increased in cells incubated with sesamin/episesamin and sesamin ($P < 0.01$) compared to DMSO control (Table 2). The lipoic acid incubation increased the fraction of 20:3n-3 ($P < 0.01$).The proportion of 20:4n-3 was significantly increased in cells incubated with lipoic acid, sesamin/episesamin and sesamin ($P < 0.01$) and slightly increased in cells incubated with 0.025 mM of genistein ($P = 0.06$).

Table 2. The FA composition (% of total FA) in cells from the point of sampling (Time 0) and after 48 h of incubation with respective treatment.

	Time 0	No treatment	DMSO control	Genistein 0.005mM	Genistein 0.025mM	Lipoic acid 0.2mM	Sesamin/epi 0.05mM	Sesamin 0.05mM
14:0	1.64	0.93 ± 0.00d	0.96 ± 0.04ad	0.98 ± 0.09ad	1.02 ± 0.06abd	1.12 ± 0.06bc	1.07 ± 0.06ac	1.10 ± 0.08bc
15:0	0.22	0.12 ± 0.00	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.15 ± 0.02	0.15 ± 0.01
16:0	14.1	12.8 ± 0.27	13.4 ± 1.00	13.0 ± 0.64	13.1 ± 0.68	13.1 ± 0.38	13.2 ± 0.87	12.7 ± 0.72
16:1n-7	1.98	1.20 ± 0.03a	1.22 ± 0.04a	1.26 ± 0.07a	1.32 ± 0.08ab	1.40 ± 0.07b	1.40 ± 0.07b	1.43 ± 0.07b
18:0	3.63	5.00 ± 0.00	5.23 ± 0.20	5.19 ± 0.06	5.31 ± 0.09	4.94 ± 0.17	5.37 ± 0.51	5.22 ± 0.46
18:1n-9	22.0	25.9 ± 0.86	24.2 ± 2.81	24.3 ± 2.79	24.1 ± 2.39	23.1 ± 2.74	24.6 ± 1.51	25.1 ± 1.75
18:1n-7	2.80	3.01 ± 0.12	2.91 ± 0.16	2.98 ± 0.07	2.98 ± 0.09	2.94 ± 0.13	2.90 ± 0.03	2.90 ± 0.03
18:2n-6	6.93	6.56 ± 0.13	6.11 ± 0.80	6.22 ± 0.82	6.40 ± 0.71	6.40 ± 0.67	6.37 ± 0.67	6.59 ± 0.78
18:3n-3	3.12	2.90 ± 0.04	2.65 ± 0.52	2.75 ± 0.51	2.86 ± 0.43	3.01 ± 0.37	2.99 ± 0.49	3.18 ± 0.57
18:4n-3	0.28	0.11 ± 0.00c	0.12 ± 0.01ac	0.12 ± 0.01ac	0.13 ± 0.02abc	0.14 ± 0.02ab	0.16 ± 0.01b	0.16 ± 0.02b
20:1n-9	2.40	3.15 ± 0.10	3.10 ± 0.05	3.05 ± 0.07	2.90 ± 0.19	2.61 ± 0.11	2.99 ± 0.15	2.89 ± 0.15
20:2n-6	0.84	1.22 ± 0.00	1.22 ± 0.06	1.21 ± 0.07	1.16 ± 0.07	1.21 ± 0.04	1.13 ± 0.07	1.12 ± 0.09
20:3n-6	0.56	0.62 ± 0.02	0.65 ± 0.05	0.65 ± 0.05	0.64 ± 0.05	0.60 ± 0.03	0.64 ± 0.02	0.63 ± 0.04
20:4n-6	1.23	1.16 ± 0.06	1.30 ± 0.16	1.37 ± 0.13	1.35 ± 0.13	1.38 ± 0.12	1.22 ± 0.03	1.16 ± 0.06
20:3n-3	0.35	0.47 ± 0.00a	0.49 ± 0.03a	0.50 ± 0.03a	0.51 ± 0.04a	0.59 ± 0.02b	0.50 ± 0.03a	0.51 ± 0.05a
20:4n-3	2.68	1.61 ± 0.01c	1.70 ± 0.09ac	1.69 ± 0.07c	1.86 ± 0.14ad	1.91 ± 0.05bd	2.03 ± 0.07b	1.94 ± 0.12bd
22:1n-9	0.17	0.22 ± 0.03	0.22 ± 0.02	0.22 ± 0.03	0.21 ± 0.03	0.17 ± 0.01	0.32 ± 0.03	0.28 ± 0.04
20:5n-3	6.73	4.22 ± 0.16	4.41 ± 0.35	4.48 ± 0.52	4.44 ± 0.38	4.83 ± 0.59	4.42 ± 0.18	4.51 ± 0.25
22:5n-3	2.60	1.99 ± 0.06	2.14 ± 0.16	2.11 ± 0.16	2.19 ± 0.15	2.29 ± 0.15	2.17 ± 0.09	2.14 ± 0.12
22:6n-3	21.6	21.8 ± 0.87	22.7 ± 1.91	22.6 ± 2.18	22.2 ± 1.69	23.4 ± 2.22	21.7 ± 0.79	21.4 ± 1.04
SAFA	20.0	19.2 ± 0.27	20.1 ± 1.22	19.6 ± 0.75	20.0 ± 0.79	19.6 ± 0.43	20.2 ± 1.43	19.5 ± 1.22
MUFA	30.7	34.7 ± 1.19	32.9 ± 2.86	33.0 ± 2.74	32.8 ± 2.32	31.4 ± 2.88	33.5 ± 1.26	33.9 ± 1.39
PUFA	47.0	42.6 ± 1.00	43.5 ± 1.49	43.7 ± 1.88	43.7 ± 1.45	45.7 ± 2.28	43.3 ± 0.55	43.3 ± 0.81
n-3 PUFA	37.4	33.1 ± 1.05	34.2 ± 2.02	34.3 ± 2.44	34.2 ± 1.95	36.1 ± 2.73	34.0 ± 0.75	33.8 ± 1.12
n-6 PUFA	9.56	9.57 ± 0.05	9.29 ± 0.53	9.45 ± 0.57	9.56 ± 0.52	9.60 ± 0.50	9.35 ± 0.58	9.49 ± 0.60
n-3/n-6	3.91	3.46 ± 0.13	3.70 ± 0.44	3.65 ± 0.49	3.59 ± 0.41	3.78 ± 0.49	3.65 ± 0.30	3.57 ± 0.33

Gene expression

In the experiment with genistein 12 hours after incubation, the relative expression of all peroxisome proliferator-activated receptors; PPARα, PPARβ and PPARγ as well as marker for elongation; ELOVL2 and β-oxidation; CPT-1 and ACO were significantly ($P < 0.05$) upregulated at the lower concentration (0.005 mM) of genistein and the relative expression of Δ-5 desaturase was significantly ($P < 0.05$) downregulated (Figure 1A). The same expression pattern could be seen after 12 hours of incubation with lipoic acid (Figure 1B). This up-regulation pattern could not be seen after incubation with the higher concentration (0.025

mM) of genistein. At the higher concentration of genistein only CD36 was significantly ($P < 0.05$) up-regulated. The same pattern of up regulated genes was seen for hepatocytes after incubation with sesamin. However, the up-regulation was only significant for ($P < 0.05$) PPARβ, PPARγ, ELOVL2 and ACO (Figure 1D). Consistently, Δ-5 desaturase was significantly ($P < 0.05$) downregulated for incubation with sesamin. Notably in the cells incubated with genistein (0.025 mM), lipoic acid or sesamin the up-regulation of ELOVL2 generated a fold change with a factor between 10.66 and 12.19 compared to the more moderate changes from 1.57 to 3.77. The only significant change was found for sesamin/episesamin after 12 h incubation was a down regulation of ACO with a factor 0.80 (Figure 1C).

Figure 1 (Lukáši, sem prosím umísti ten obrázek i s popiskem, co je na konci článku)

After 48 hours of incubation with genistein no effect could be seen with none of the concentrations on elongation, desaturation or β-oxidation markers. The marker for uptake; CD36 as well as the transcriptions factors PPARα and PPARγ were all significantly downregulated ($P < 0.05$) at lower concentrations of genistein (Figure 2A). However, the down regulation was moderate with factors ranging from a factor 0.49 to 0.74. Likewise was a fairly moderate but significant up regulation of PPARβ seen after the same incubation interval with the higher concentration of genistein. For hepatocytes incubated with lipoic acid, sesamin/episesamin and sesamin all genes involved in uptake, elongation, desaturation and β-oxidation were significantly ($P < 0.05$) up-regulated after 48 hours except for the transcription factors. Treatment with lipoic acid caused a significant ($P < 0.05$) downregulation of transcription factors PPARα and PPARβ but a significant ($P < 0.05$) up-regulation of PPARγ (Figure 2B). For hepatocytes incubated with sesamin/episesamin (Figure 2C) transcription factors PPARα and PPARβ were significantly upregulated ($P < 0.05$), but sesamin incubation only resulted in an upregulation of transcription factor PPARγ (Figure 2D).

Figure 2 (Lukáši, sem prosím umísti ten obrázek i s popiskem, co je na konci článku)

Discussion

The aim of this study was to evaluate the effect of bioactive compounds, known to act as antioxidants and/or influence lipid homeostasis in mammals, on the underlying lipid related gene expression in Atlantic salmon hepatocytes and the outcome fatty acid profile.

Atlantic salmon hepatocytes are an established *in vitro* system and have been used in a range of lipid metabolic studies (Kjær et al., 2008; Krøvel et al., 2010; Torstensen et al., 2004; Trattner et al., 2008a; Zhou et al., 2010). However, the results from this study are needed to be seen in the perspective that the limitations are in the *in vitro* environment.

During the trial Atlantic salmon hepatocytes incubated with genistein did not show any significant difference in FA composition compared to control incubations (both with and without DMSO). However, hepatocytes incubated with lipoic acid, sesamin/episesamin and sesamin showed a significant increase in relative concentration of 20:4n-3. Lipoic acid supplement also increased the relative concentration of 20:3n-3. On the contrary to the study by Trattner et al. (2008a) no effects were observed in the present study on DHA level. One could speculate that it might be caused due to the bigger size of fish used in our experiment. It has been shown that the synthesis of DHA vary during the production cycle with the lowest activity in the seawater phase in salmon (Zheng et al., 2005). It is also possible that different feeding regime or physiological status of the fish used for the isolation of hepatocytes could play an important role.

We could see that all the four tested compounds influenced expression of genes involved in LCPUFA biosynthesis. After 12 hours of incubation with lipoic acid, sesamin and low concentrations of genistein the transcription factors PPARβ and γ were up-regulated. PPARα was also up-regulated when incubated with lipoic acid and low concentrations of genistein. This indicates an increased transcription of target genes. PPARα, β and γ are acting as sensors for any change in levels of FAs by answering to ligand binding. By doing so the PPARs target gene transcription influencing lipid homeostasis (Burri et al., 2010). PPARα has shown to be important regulator of FA metabolism in mice, by induction of genes coding for the fatty acid transporter CD36. Another PPARα target gene is CPT1 coding for a protein regulating the FA transport over the membrane of the mitochondria. PPARγ has been shown to be involved in the regulation of FA metabolism and adipocyte differentiation in salmon liver (Ruyter et al., 1997; Vegusdal et al., 2003). PPARα is also known to induce Δ-6 desaturase enzymatic activity by an increased rate of Δ-6 desaturase gene transcription (He et al., 2002).

Both sesamin/episesamin and sesamin as well as lipoic acid increased the gene expression of the long chain fatty acid transporter, CD36, which is located in the plasma membrane after 48 hours of incubation. This indicates that these three bioactive compounds increase the hepatic uptake of fatty acids from the blood to place in disposal as substrates for coming fatty acid oxidation.

The elongase ELOVL2 was highly up-regulated coupled with a marked down-regulation of Δ-5-fatty acid desaturase after 12 hours of incubation with lipoic acid, sesamin and low concentrations of genistein. The downregulation of desaturases has been detected before in salmon hepatocytes incubated with sesamin/episesamin (Trattner et al., 2008a), however in connection to increased amount of radio-labelled DHA. The expression of ELOVL2 seems to be the most sensitive lipid related gene for the bioactive compounds tested in this study, in agreement with Morais et al. (2009) who suggested ELOVL2 to be more prominent in salmon in contrast to rats where ELOVL5 is the most responding to dietary changes. ELOVL2 is involved in the elongation of 20 and 22 carbon FA towards longer FA (Morais et al., 2009). In contrast, we did not see increase in the PUFA longer than C20 in any of the treatments. Interestingly, there was also quite different expression pattern between sesamin and sesamin/episesamin. Different effects of pure sesamin and episesamin was already reported by Kushiro et al. (2002) in studies with rats and it was also suggested by our study with carp (Mraz et al., 2010). After 48 hours of incubation with lipoic acid, sesamin/episesamin or sesamin an up regulation all the genes chosen as markers (ELOVL5a elongase, Δ-5-fatty acid desaturase, ELOVL2 elongase and Δ-6-fatty acid desaturase) for the biosynthesis of LCPUFAs were seen suggesting that these compounds are potent modulators of the LCPUFA pathway.

CPT1 and ACO were up-regulated after addition of lipoic acid and low concentrations of genistein to the growth medium and ACO was also up-regulated after adding sesamin to the medium, indicating a potential increase in β-oxidation. This is in agreement with our previous study, which shown increased levels of β-oxidation products after addition of sesamin/episesamin to hepatocytes (Trattner et al., 2008a). Sesamin is also used as a weight reducing compound in human medicine due to its capacity to increase β-oxidation. Genistein affected the CPT1 gene involved in mitochondrial β-oxidation. Therefore, one could expect decrease in FA (e.g. SAFA) commonly preferred as substrate for β-oxidation, however this was not detected in any of the treatments.

The addition of bioactive compounds to salmon hepatocytes needs further studies. On one hand studies need to be made where the fish will be fed vegetable oil diets prior to hepatocytes preparation and on the other hand further gene expression studies need to be performed examining hepatic genes coding for the metabolism of bioactive compounds.

In conclusion, several effects on FA composition and genes related to LCPUFA pathway were observed in the present study. We could see that all the four tested compounds influenced expression of genes involved in LCPUFA biosynthesis. The results suggest that these compounds are potent modulators of HUFA biosynthetic pathway in fish. This may have implications for the future use of selected bioactive compounds in aqua-feeds to improve lipid quality of fish fed vegetable oil based diets.

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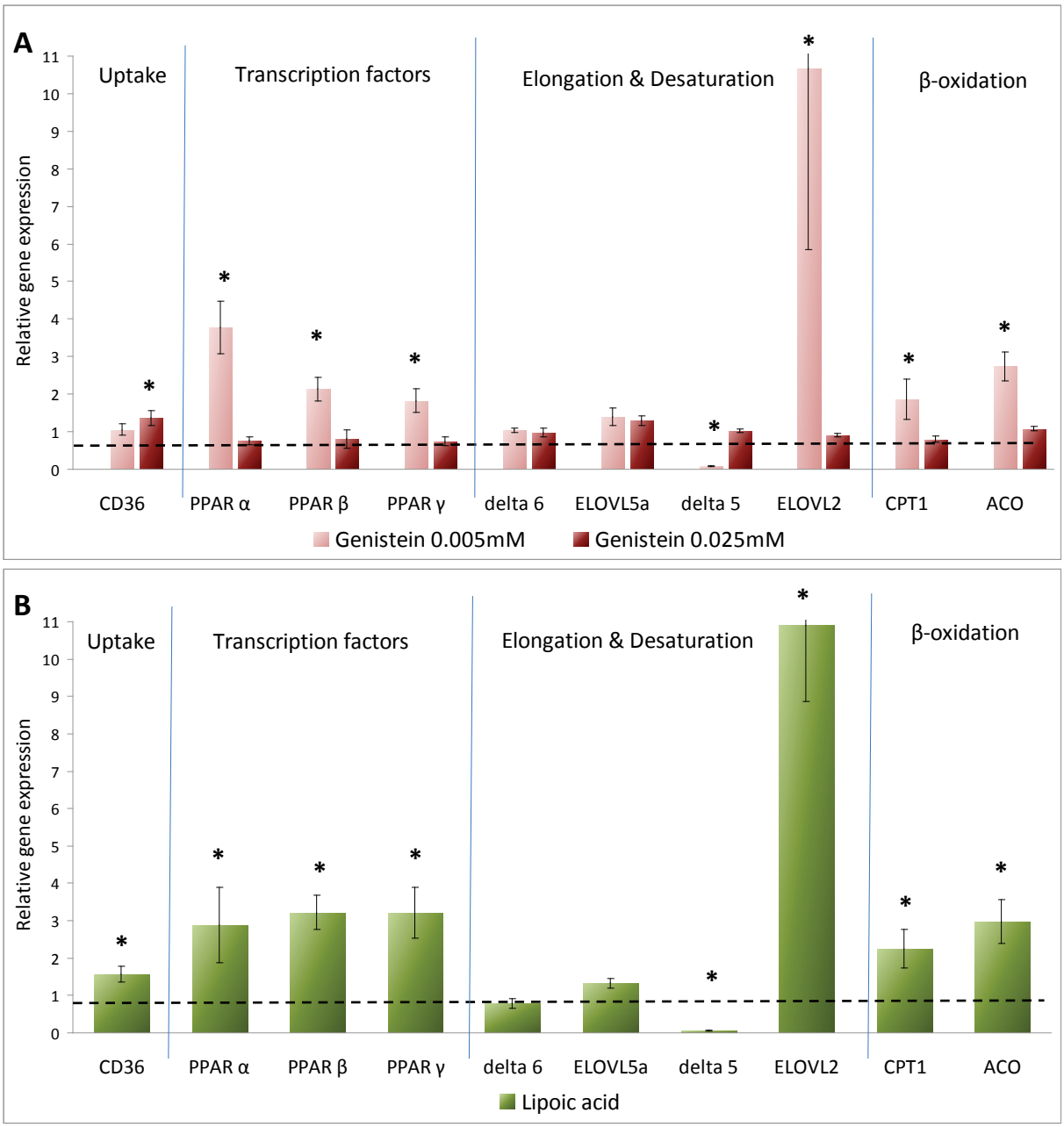
Figure 1. The gene expression in hepatocytes of Atlantic salmon 12 hours after exposure to (A) two different concentrations of Genistein (0.005 mM and 0.025 mM), (B) Lipoic acid, (C) Episesamin and (D) Sesamin. The relative gene expression was calculated according to $2^{-\Delta\Delta Ct}$ method using RPL (RNA polymerase 2) as a reference gene and normalized against control samples treated with DMSO.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CPT1
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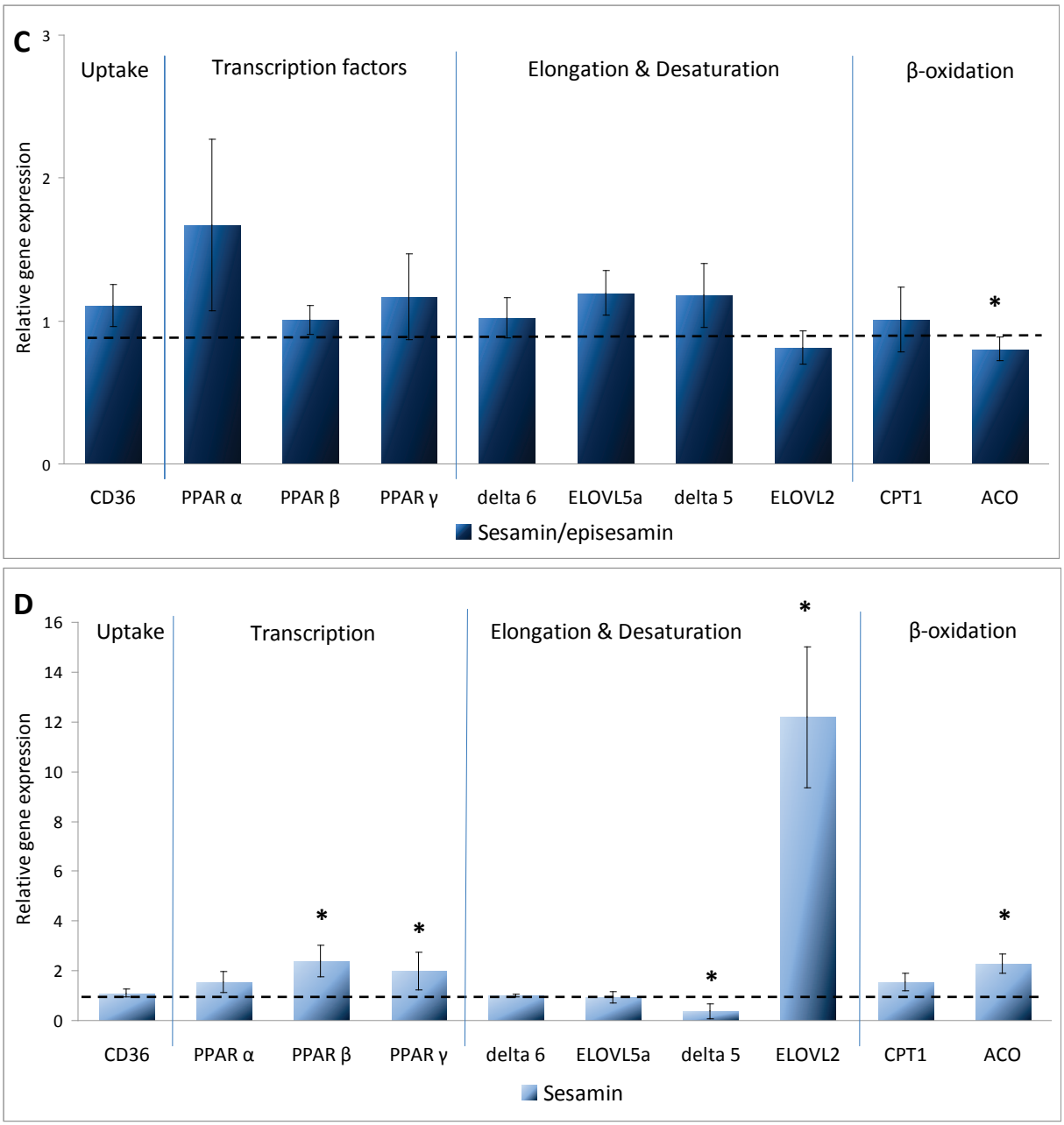
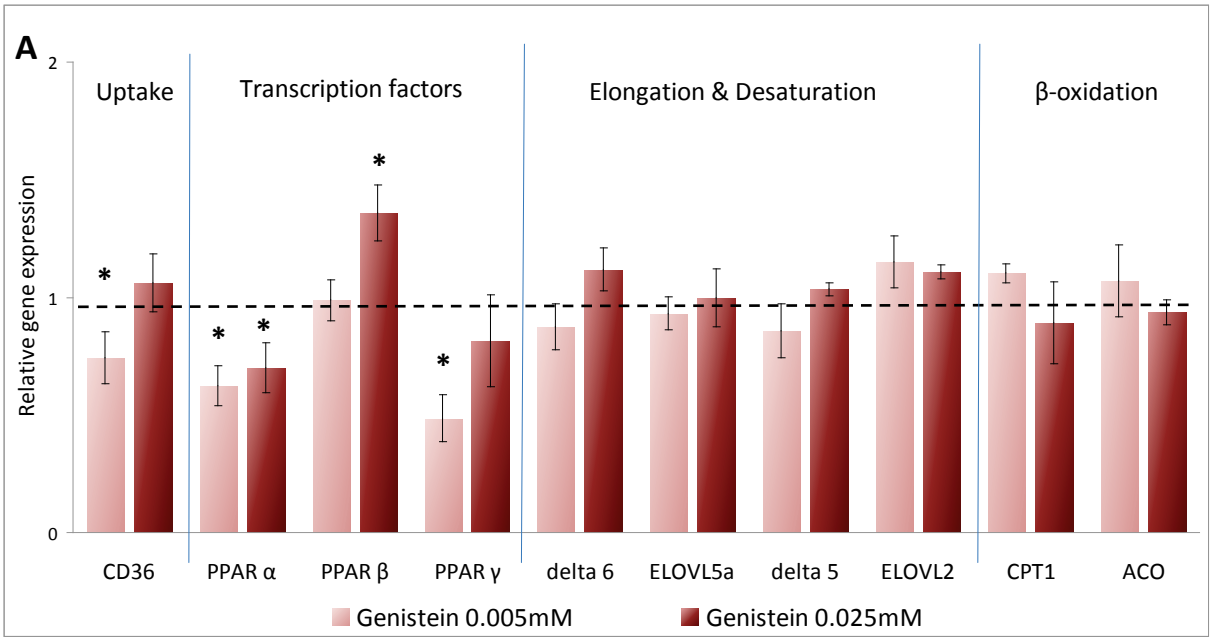
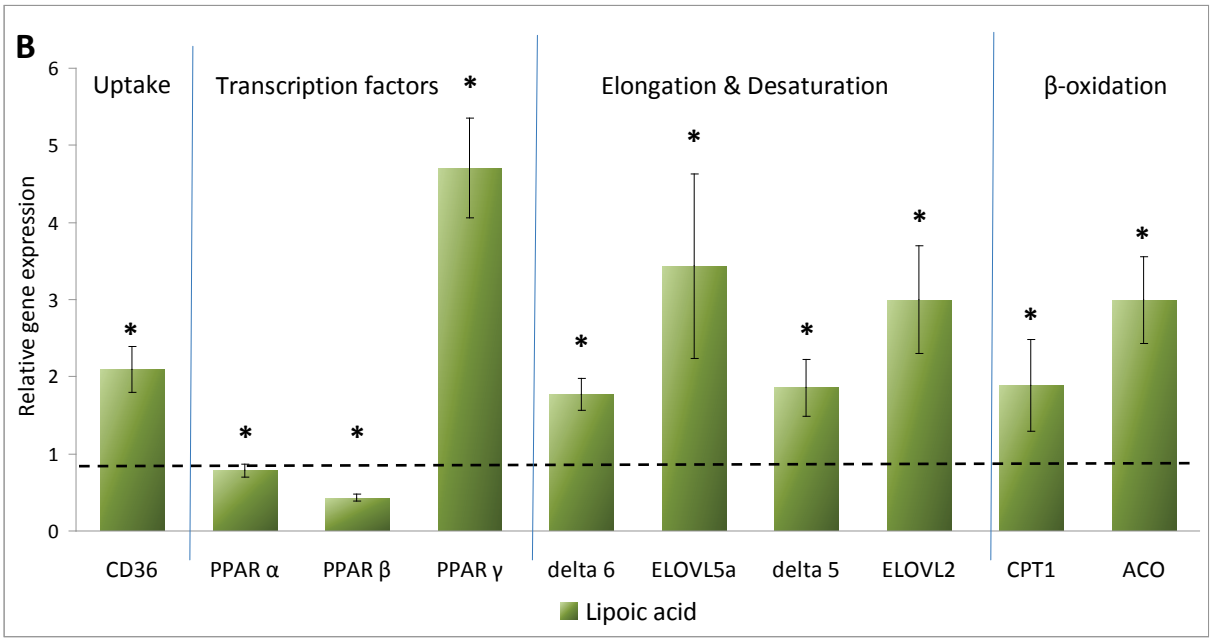
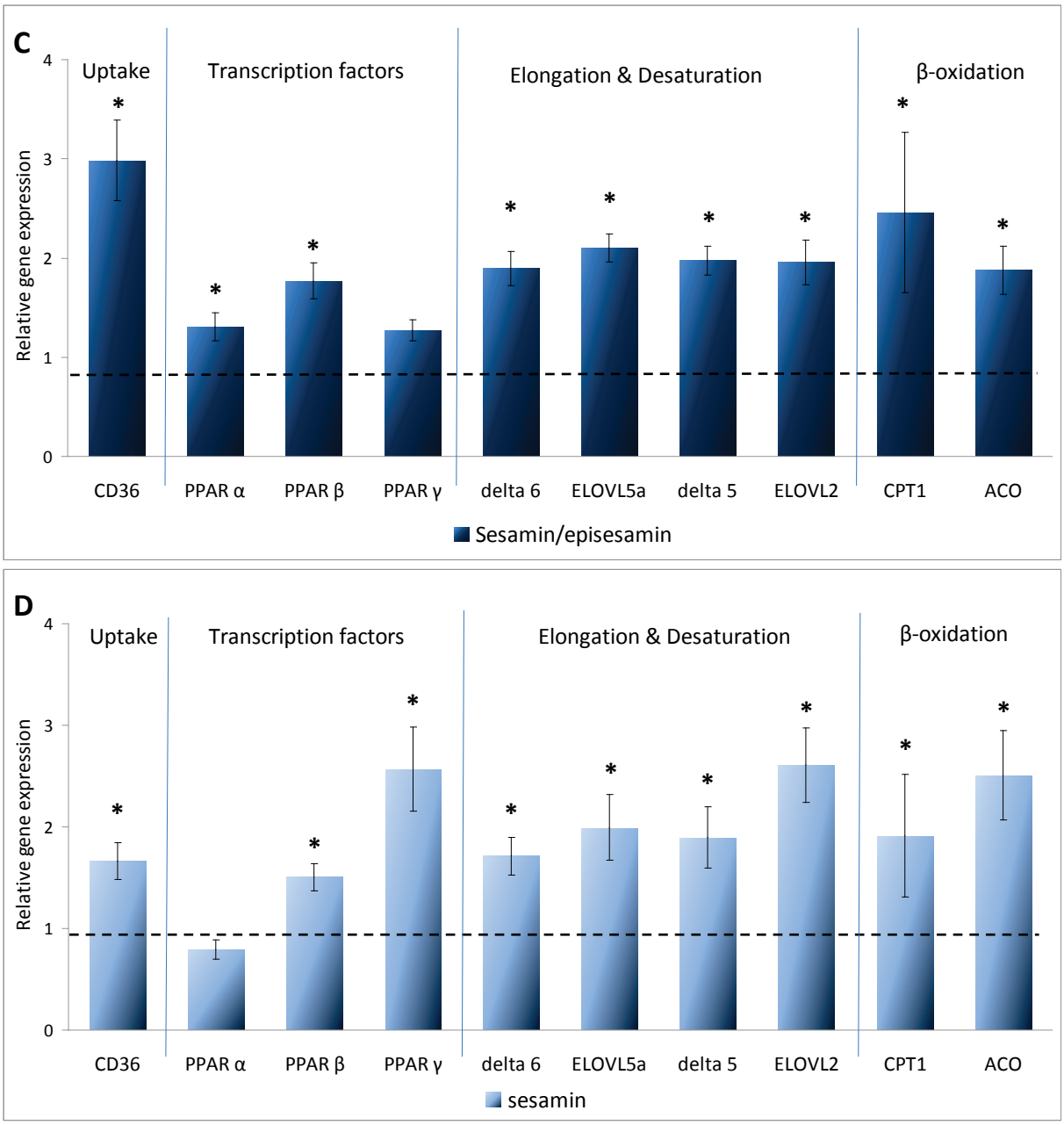


Abbreviations: CD36, CD36 thrombospondin receptor; PPAR, peroxisome proliferator-activated receptor, delta 6, Δ-6-fatty acid desaturase; ELOVL5a, elovl5a elongase; delta 5, Δ-5-fatty acid desaturase; ELOVL2, elovl2 elongase; CPT1, Carnitine palmitoyltransferase 1; ACO, acyl-coenzyme A oxidase.

Figure 2. The gene expression in hepatocytes of Atlantic salmon 48 hours after exposure to (A) two different concentrations of Genistein (0.005 mM and 0.025 mM), (B) Lipoic acid, (C) Episesamin and (D) Sesamin. The relative gene expression was calculated according to $2^{-\Delta\Delta Ct}$ method RPL (RNA polymerase 2) as a housekeeping gene and normalized against control samples treated with DMSO.







Abbreviations: CD36, CD36 thrombospondin receptor; PPAR, peroxisome proliferator-activated receptor, delta 6, Δ-6-fatty acid desaturase; ELOVL5a, elovl5a elongase; delta 5, Δ-5-fatty acid desaturase; ELOVL2, elovl2 elongase; CPT1, Carnitine palmitoyltransferase 1; ACO, acyl-coenzyme A oxidase.

Chapter 6

General Discussion
English Summary
Czech Summary
Acknowlodgements
List of Publications
Training and Supervision Plan during Study
Curriculum Vitae

General Discussion

There is a large body of evidence that n-3 fatty acids, especially EPA and DHA are beneficial for human health, especially for cardiovascular system. The sources of n-3 FA, including EPA and DHA, are scarce in diet consumed by Czech population. Therefore the overall aim of this thesis was to evaluate factors influencing lipid content and composition in common carp, which is the major cultured fish in the Czech Republic. We wanted to develop a sustainable way for improvement of carp muscle lipid quality to become a requested healthy and local product for prevention and treatment of cardiovascular diseases. Following specific topics were studied and are further discussed in details: 1) Distribution and composition of lipids within the carp fillet. 2) How the lipid quality is influenced by the genetic background of the carps. 3) Examination whether the lipid composition of common carp muscle can be improved by different rearing strategies in ponds and become an important source of n-3 highly unsaturated fatty acids for human nutrition. 4) Using biologically active compounds to modulate the fish metabolism to synthesize more n-3 HUFA from ALA.

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 (Johnston et al., 1977; Rome et al., 1993).

(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).

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.

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. 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.

These results have several implications. It has to be kept in mind that the lipid composition is not equally distributed in the fish fillet and therefore the sampling location has a high impact if one wants to compare different samples. Further, it has to be count with the fact that samples of carp flesh with the higher lipid content usually have the lower relative percentage of PUFA and higher MUFA. So if one wants to compare effects of different breeds, diets, bioactive compounds etc. on FA composition needs to use fish of approximately the same weight and lipid content or normalise the data against the lipid content. The results have also practical application for processing of possible fish products with low lipid content and good FA composition.

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., 2004; Prikryl, 1984). Our data show (Paper I, III + 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 sampled from ponds occured 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. 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 et al., 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, 1996; Henderson, 1996). This could be probably altered by a change in feed composition by other vegetable sources than cereals.

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 was plausibly to be ascribed to differences in the lipid content and consequently to different proportion between the two lipid fractions and their different FA composition. 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 more distant, e.g.: the two subspecies of common carp *C.c. carpio* and *C.c. haematopterus*. The other limitation is the use of semi-intensive technique for such experiment. The first reason is that it is not possible to separate possible genetic differences in lipid metabolism and behavioural differences among the crossbreeds. The second reason is that when using supplemental feed based on

cereals the fish do not have enough ALA (n-3 HUFA precursors) in the diet to show any difference in ability to biosynthesise n-3 HUFA.

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 central European population. We conclude 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.

We showed in paper III that lipid composition in carp flesh is strongly influenced by the pond production system. We compared flesh lipid quality in common carp from an extensive system with low carp density and without feed supplementation with two semi-intensive systems with higher carp densities based on either cereal supplementation or supplementary pellets containing rapeseed cake. The fish from the extensive system were characterized by a high content of n-3 HUFA, especially EPA and DHA. The fish supplemented with rapeseed pellets had a moderate n-3 HUFA level, while those supplemented with cereals were characterized by a high content of oleic acid and low level of n-3 HUFA.

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.

The aim of the study in Paper II was to investigate if diet supplemented with the lignan sesamin, a boactive compound from sesame seed, has an effect on fatty acid composition in common carp muscle as was previously seen in salmonids (Trattner et al., 2008a; Trattner et al., 2008b). The results of FA composition did not show such effect.

In Paper II, we discussed several explanations for the discrepancy between the responses in fatty acid alterations.

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., 2008b). 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 of the fish also need to be considered as various response on such compound can be expected depending on the maturation of metabolism of the fish, thereby the effect of sesamin.

We found in this study that carps supplemented with sesamin showed higher EROD activity and higher content of total CYP P450, as reported also in rainbow trout liver (Trattner et al., 2008b). 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.

We repeated the experiment using juveniles of common carp and diet with very low level of n-3 HUFA and to test if any effects of sesamin other than in study II can be recognised (unpublished results). However, there was not any effect on FA composition observed either.

In conclusion, we have not been able to recognise of HUFA biosynthesis in carp. 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 is possible that other bioactive compounds which influence n-3 HUFA biosynthesis will be identified and used in fish culture.

However, in order to identify such natural bioactive compounds or optimized dietary composition, we need first to understand more of the regulation of n-3 HUFA biosynthetic pathway. The regulation of the pathway is not fully understood in common carp and therefore it is not known if it is possible to utilize the ability of carp to produce n-3 HUFA. Our anticipation is that by the understanding molecular background we may identify tools for optimization of the LCPUFA biosynthesis. By so doing enable an efficient sustainable production of common carp with higher nutritional value.

In paper IV, Screening for other bioactive compounds which could modulate the fish metabolism to induce synthesis of n-3 HUFA from ALA was performed. Several compounds were selected (pure sesamin, sesamin/episesamin, lipoic acid, genistein) and their effects on fatty acid composition and gene expression were studied *in vitro* using hepatocytes from Atlantic salmon. Atlantic salmon hepatocytes were chosen for several reasons. Firstly, it is a well established model system to study fish lipid metabolism. The system is well developed, genes related to HUFA pathway are already identified and characterised in Atlantic salmon etc. We have with the Norwegian lab at NOFIMA, where the study was performed. This research environment is one of the world leading laboratories in lipid studies both *in vivo* and *in vitro*. Our previous cell culture study with the use of sesamin/episesamin by (Trattner et al., 2008a) was performed in this lab. We are aware of limitations in interpretation of results obtained from this kind of models. However, it enables us to screen for high number of compounds at the same time with much lower costs and time compared with *in vivo* system and select the compounds with the strong effects for further experiments. In addition, less experimental animals are needed which is of large importance.

Lipoic acid was chosen because it has been shown to increase level of EPA in pacu muscle (Trattner et al., 2007). Genistein is known to influence lipid metabolism in rats (Takahashi et al., 2009). Pure sesamin and equimixture of sesamin/episesamin were selected to see if there is a difference in effects between the sesamin only and equimixture as indicated in paper II.

Several effects on FA composition and genes related to HUFA pathway were observed in the present study. Lipoic acid, sesamin/episesamin and sesamin increased level of 20:4n-3. Lipoic acid increased also level of 20:3n-3. There was no impact of genistein observed on FA composition. We have not recognised any increase of DHA in sesamin/episesamin treatment which was previously reported by (Trattner et al., 2008a). We could see that all the four tested compounds influenced expression of genes involved in HUFA biosynthesis. After 12 hours of incubation, the highest effect was seen in very strong up-regulation of ELOVL2. Interestingly, there was also quite different expression pattern between sesamin and

sesamin/episesamin. After 48 hours of incubation, there was a strong up-regulation of all the elongases, desaturases, CD36 (marker for uptake), markers of β-oxidation as well as PPARs seen in hepatocytes incubated with lipoic acid, sesamin and sesamin/episesamin. These results show that these compounds are potent modulators of HUFA biosynthetic pathway in fish. This may have implications for future use of selected bioactive compounds in aqua-feeds to improve lipid quality of fish fed vegetable oil based diets.

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English Summary

Improvement of fatty acid composition in common carp (*Cyprinus carpio*)

Jan Mráz

There is a large body of evidence that n-3 fatty acids, especially EPA and DHA are beneficial for human health, especially for cardiovascular system. The sources of n-3 FA, including EPA and DHA, are scarce in diet consumed by Czech population. Therefore the overall aim of this thesis was to evaluate factors influencing lipid content and composition in common carp, which is the major cultured fish in the Czech Republic, and to find the long term sustainable way how to improve carp muscle lipid quality in order to be used as a local healthy product for prevention and treatment of cardiovascular diseases. Following specific topics were studied 1) Distribution and composition of lipids within the carp fillet. 2) How the lipid quality is influenced by the genetic background of the carps. 3) Examination whether the lipid composition of common carp muscle can be improved by different rearing strategies in ponds and become an important source of n-3 highly unsaturated fatty acids for human nutrition. 4) Using biologically active compounds to modulate the fish metabolism to synthesize more n-3 HUFA from ALA.

The results showed that lipid content and composition differed strongly among different parts of common carp fillet. The lowest lipid content was found in dorsal white muscle, medium in red muscle, whereas the highest was found in abdominal wall. 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 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.

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. We showed that lipid composition in carp flesh is strongly influenced by the pond production system. Flesh lipid quality in common carp from an extensive system with low carp density and without feed supplementation was compared with two semi-intensive systems with higher carp densities based on either cereal supplementation or supplementary pellets containing rapeseed cake. The fish from the extensive system were characterized by a high content of n-3 HUFA, especially EPA and DHA. The fish supplemented with rapeseed pellets had a moderate n-3 HUFA level, while those supplemented with cereals were characterized by a high content of oleic acid and low level of n-3 HUFA.

Effects of bioactive compound sesamin on FA composition and global gene expression were tested *in vivo* in common carp. 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.

Screening for other bioactive compounds which could modulate the fish metabolism to synthesize more n-3 HUFA from ALA was performed. Several compounds were selected (pure sesamin, sesamin/episesamin, lipoic acid, genistein) and their effects on fatty acid composition and gene expression were studied *in vitro* using hepatocytes from Atlantic salmon (*Salmo salar* L.). Several effects on FA composition and genes related to HUFA pathway were observed in the present study. Results showed that these compounds are potent modulators of HUFA biosynthetic pathway in fish. This may have implications for the future use of selected bioactive compounds in aqua-feeds to improve lipid quality of fish fed vegetable oil based diets.

Czech Summary

Vylepšení kompozice mastných kyselin u kapra obecného (*Cyprinus carpio*)

Jan Mráz

Existuje velké množství důkazů, že n-3 polynenasycené mastné kyseliny (n-3 PUFA), zvláště EPA a DHA, jsou prospěšné pro lidské zdraví, zejména pro kardiovaskulární systém. Zdroje n-3 PUFA, včetně EPA a DHA, jsou ve stravě konzumavané českou populací relativně vzácné. Proto bylo cílem této práce zhodnotit faktory, které ovlivňují obsah a složení lipidů u kapra obecného, což je hlavní chovaná ryba v České republice, a najít dlouhodobě udržitelný způsob, jak zlepšit kvalitu lipidů v kapří svalovině tak, aby mohla být používána jako lokální zdravý produkt pro prevenci a léčbu kardiovaskulárních chorob. V rámci práce byla zkoumána tato konkrétní témata 1) Distribuce a složení lipidů v kapřím filetu. 2) Jak je kvalita lipidů ovlivněna genetickým pozadím kapra. 3) Zda lze zlepšit lipidové složení kapřího filetu různými strategiemi chovu v rybnících a zda se kapr může stát důležitým zdrojem n-3 PUFA pro lidskou výživu. 4) Využití biologicky aktivních látek k modulaci metabolismu ryb tak, aby syntetizovaly více EPA a DHA z kyseliny alfa-linolenové.

Výsledky ukázaly, že obsah a složení lipidů se výrazně liší mezi různými částmi kapřího filetu. Nejnižší obsah lipidů byl nalezen v bílé hřbetní svalovině, střední v červené svalovině, zatímco nejvyšší byl nalezen ve stěně. Abdominální stěna s nejvyšším obsah lipidů byla charakteristická velkým obsahem triacylglycerolů, zatímco bílá svalovina měla nejvyšší podíl fosfolipidů. Složení mastných kyselin se mezi různými částmi výrazně lišilo v závislosti na obsahu tuku a poměru mezi fosfolipidy a triacylglyceroly. Bílá svalovina, která je nejméně tučnou částí, byla typická velkým podílem fosfolipidů, n-3 vysoce nenasycených mastných kyselin (n-3 HUFA) a poměrem n-3/n-6 = 1,1. Zatímco abdominální stěna byl bohatá na mononenasycené mastné kyseliny a měla nižší poměr n-3/n-6 = 0,5.

Čtyři kříženci kapra, kteří byli analyzováni v naší studii, měli podobnou skladbu mastných kyselin v bílé hřbetní svalovině. Rozdíly ve složení mastných kyselin pozorované v celkovém lipidu byly připsány rozdílům v obsahu celkového tuku a následně různému poměru mezi fosfolipidy a triacylglyceroly.

Došli jsme k závěru, že kapr má potenciál stát se atraktivní rybou z hlediska obsahu a složení lipidů. Nutriční hodnota kapřího filetu je vysoká, vzhledem k množství n-3 PUFA ve stravě středoevropské populace. Ukázali jsme, že složení lipidů v mase kapra je silně ovlivněno produkčním systémem chovu. Porovnali jsme kvalitu lipidů v mase kapra z extenzivního chovu s nízkou hustotou obsádky bez přikrmování ve srovnání se dvěma polo-intenzivními systémy s vyšší hustotou obsádky s přikrmováním obilovinami nebo krmnou směsí obsahující řepkové výlisky. Ryby z extenzivního způsobu chovu byly charakterizovány vysokým obsahem n-3 HUFA, zvláště EPA a DHA. Ryby přikrmované krmivem s řepkovými výlisky měly střední množství n-3 HUFA, zatímco ryby přikrmované obilovinami se vyznačovaly vysokým obsahem kyseliny olejové a nízkou úrovní n-3 HUFA.

Dále jsme u kapra testovali účinky bioaktivní látky sesaminu na složení mastných kyselin a genovou expresi. V této studii neměl přídavek sesaminu vliv na složení mastných kyselin ve svalovině kapra. Sesamin zvýšil celkový obsah cytochromu P450 v mikrozomech hepatopankreatu stejně jako EROD aktivitu a změnil v hepatopankreatu expresi 662 genů. Nicméně, jsme nebyli schopni definovat žádné změny v metabolismu lipidů. Dohromady tyto výsledky ukazují, že sesamin je u kapra neúčinnou látkou k dosažení změněného složení lipidů.

Následně byl proveden screening pro další bioaktivní látky, které by mohly ovlivnit metabolismus ryb tak, aby syntetizovaly více n-3 HUFA z kyseliny alfa-linolenové. Bylo vybráno několik komponent (čistý sesamin, sesamin/episesamin, kyselina lipoová a genistein) a byl *in vitro* studován jejich vliv na složení mastných kyselin a expresi genů s použitím lososích hepatocytů (*Salmo salar* L.). Tyto bioaktivní látky měly vliv na složení mastných kyselin a expresi genů spojených se syntézou HUFA. Výsledky ukázaly, že tyto látky jsou u ryb účinnými modulátory HUFA biosyntézy. To může mít důsledky pro budoucí využití vybraných bioaktivních složek v krmivech pro zlepšení kvality lipidů ryb krmených potravou na bázi rostlinných olejů.

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Adámková, V., Hubácek, J.A., **Mráz, J.**, Králová Lesná, I., Pickova, J., Kozák, P., Suchánek, P., Kacer, P., Stávek, P., Skibová, J., 2011. Effect of increased consumption of carp flesh on lipid parameters in pacients after cardiovascular revasularization. (manuscript)

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Application of methodologies, patents, pilot plant, verified technology

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Abstracts and conference proceedings

Adámková, V., Hubácek, J.A., **Mráz, J.**, Králová Lesná, I., Pickova, J., Kozák, P., Suchánek, P., Kačer, P., Stávek, P., Skibová, J., 2010. The effect of increased consumption of carp fillet on blood lipid parameters in patients after cardiovascular revascularization. In: Adámková, V., Hubácek, J.A. (Eds), Nutrition – an integral part of treatment of serious diseases. November, Poděbrady, Czech Republic, ISBN 978-80-7394-238-0. (oral presentation)

Mráz, J., Kozák, P., Pickova, J., 2010. Quality of common carp fillet in dependence on food chain. In: Adámková, V., Hubácek, J.A. (Eds), Nutrition – an integral part of treatment of serious diseases. November, Podebrady, Czech Republic. ISBN 978-80-7394-238-0. (oral presentation)

Žlábek, V., Trattner, S., **Mráz, J.**, Brännäs, E., Samuelsen, T., Pickova, J., 2010. The effects of including sesamin in fish diets on cytochrome enzymes. In: Aquaculture Europe 2010. Porto, Portugal, pp. 1437–1438. (oral presentation)

Mráz, J., Kozák, P., Pickova, J., 2009. Improvement of fatty acid composition in common carp (*Cyprinus carpio*) muscle. In: Nutrition – an integral part of treatment of serious diseases. November, České Budějovice, Czech Republic. (oral presentation)

Mráz, J., Kozák, P., Pickova, J., 2009. Improvement of fatty acid composition in common carp (*Cyprinus carpio*) muscle. In: 7th Euro Fed Lipid Congress, Graz, Austria.

Mráz, J., Pickova, J., 2008. Fatty acid composition in different parts of farmed common carp (*Cyprinus carpio* L.) muscle. In: Kamler E., Dabrowski K. (Eds), Ressource management, Aquaculture Europe, Krakow, 2008. ISBN 978-83-60111-30-7. (poster presentation)

Kouřil, J., **Mráz, J.**, Hamáčková, J., Barth, T., 2007. Hormonal induction of tench (*Tinca tinca* L.) with the same treatments at two sequential reproductive seasons. In: The 8th International Symposium on Reproductive Physiology of Fish. June 3–8, St. Malo, France. (poster presentation)

Training and Supervision Plan during Study

Name	Jan Mráz
Research department	2006–2009 – Department of Aquaculture of RIFCH 2009–2011 – Laboratory Ethology and Nutrition of Fish and Crayfish of FFPW
Main supervisor	Prof. Jana Picková, Ph.D.
Period	1st October 2007 until 30th September 2011

Ph.D. courses	**Year**
Biostatistic	2008
Fish nutrition	2009
English language	2009
Fish genetics	2009
Fish reproduction	2009

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2008
Seminar days of RIFCH and FFPW	2009
Half time seminar at SLU	2010
Licentiate seminar at SLU	2011

International conferences	**Year**
Aquaculture Europe, Krakow, Fatty acid composition in different parts of farmed common carp (*Cyprinus carpio* L.) muscle. (poster presentation)	2008
7th Euro Fed Lipid congress, Graz, Improvement of fatty acid composition in common carp (*Cyprinus carpio*) muscle. (oral presentation)	2009
Nutrition – an integral part of treatment of serious diseases. Improvement of fatty acid composition in common carp (*Cyprinus carpio*) muscle. (oral presentation)	2009
Nutrition – an integral part of treatment of serious diseases. Quality of common carp fillet in dependence on food chain. (oral presentation)	2010

Foreign stays during Ph.D. study at RIFCH and FFPW	year
Prof. Jana Picková, Swedish University of Agricultural Sciences, Department of Food Science, Sweden. (24 months, Lipid analyses, bioactive compounds, gene expression)	2007–2011
Prof. Bente Ruyter, Nofima, Norway. (cell cultures)	2010

Curriculum Vitae

Personal Information
Surname: Mráz
First name: Jan
Title: Dipl.-Ing.; Ph. Lic.
Born: 17th February, 1983
Nationality: Czech
Martial Status: Married

Education
Dipl.-Ing. – University of South Bohemia in České Budějovice, Agricultural Faculty, České Budějovice, Czech Rebublic, specialization fishery, 2002–2007
Ph. Lic. – Swedish University of Agricultural Sciences, Department of Food Science, Sweden. Thesis: Lipid quality of common carp (*Cyprinus carpio*) in pond culture. 2007–2011

Professional experience
2007–2011 – Ph.D. student – University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Laboratory of Ethology and Nutrition of Fish and Crayfish, Czech Republic
2008 – present – Ph.D. student, Swedish University of Agricultural Sciences, Department of Food Science, Sweden

Ph.D. courses
Biostatistic, Fish nutrition, Fish reproduction, Fish genetics, English language, Physical and biochemical methods for analysis of fish as food, Functional foods for human health, Scientific writing within food science, In vitro techniques in studies of reproductive toxicology, Lipids in evolution, Functional genomics, Frontlines in omics, Gene expression and genotyping.

Specialization
Fish nutrition and lipid metabolism
Knowledge of languages: English, German, Swedish (basics)

Foreign stays during Ph.D. study at RIFCH and FFPW
Prof. Jana Picková, Swedish University of Agricultural Sciences, Department of Food Science, Sweden. (24 months, Lipid analyses, bioactive compounds, gene expression)

Prof. Bente Ruyter, Nofima, Norway. (cell cultures)