The Effect of Diet and the Masou Salmon Delta5-Desaturase Transgene on Delta6-Desaturase and Stearoyl-CoA Desaturase Gene Expression and N-3 Fatty Acid Level in Common Carp (*Cyprinus carpio*).

by

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Abstract

The omega-3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) play important roles in human health. Transgenic technology and diet regulation elevated the omega-3 fatty acid levels in common carp (*Cyprinus carpio*). Masou salmon Δ5-desaturase-like gene driven by the common carp β-actin promoter (D5D) was transferred into common carp that were later fed two different diets (a formulated diet containing high fatty acid substrates (C18:2n-6/C18:3n-3), high polyunsaturated fatty acid (PUFA) but low highly unsaturated fatty acid (HUFA), and a commercial diet containing low fatty acid substrates, but high PUFA and HUFA to determine its effect on the transcription level of endogenous Δ6-desaturase-like gene (D6D) and stearoyl-CoA desaturase（SCD）in four tissues, muscle, liver, brain and gonad and omega-3 fatty acid content in muscle. The transgene was randomly distributed in all four tissues screened, liver, brain, muscle, and gonad with 8.3%, 10%, 21.7%, and 26.7% of individuals transgenic, respectively, for these 4 tissues. In some cases, the transgene was found in 2 tissues (1.7-5.0%), but no fish were detected that were transgenic in 3 tissues. Quantitative reverse-transcriptase PCR (qRT-PCR) of D6D gene expression in the common carp fed commercial diet tissues showed a rank high to low order of brain, liver, gonad and muscle. And SCD gene expression in the common carp fed commercial diet tissues showed a rank high to low order of liver, brain, gonad and

muscle. The D6D and SCD mRNA level were 8.5-fold and 9.1-fold higher in non-transgenic fish liver fed commercial diet than that fed formulated diet, respectively. The SCD mRNA level was 2.2-fold higher in non-transgenic fish muscle fed formulated diet than that fed commercial diet. For the fish fed commercial diet: 1) D6D and SCD mRNA level in muscle of transgenic fish were up-regulated ($P<0.05$) 12.7-fold and 17.9-fold, respectively, compared to that of non-transgenic fish. 2) D6D mRNA level in the gonad of transgenic fish was up-regulated 6.9-fold ($P<0.05$) compared to that of non-transgenic fish. 3) In contrast, D6D and SCD mRNA level in the brain of transgenic fish were dramatically down-regulated 50.2-fold and 16.7-fold ($P<0.05$), respectively, compared to non-transgenic fish. 4) D6D and SCD mRNA level in liver of transgenic fish was down-regulated ($P<0.05$) 5.4-fold and 2.4-fold, respectively, compared to non-transgenic fish. For the fish fed formulated diet: 1) D6D and SCD mRNA level in muscle of transgenic fish were up-regulated ($P<0.05$) 41.5-fold and 8.9-fold, respectively, compared to that of non-transgenic fish. 2) D6D and SCD were also up-regulated in liver of transgenic fish, 6.0-fold and 3.3-fold ($P<0.05$), respectively, compared to non-transgenic fish. 3) In contrast, D6D and SCD mRNA level in the gonad of transgenic fish were down-regulated 5.5-fold ($P<0.05$) and 12.4-fold ($P< 0.05$) respectively compared to that of non-transgenic fish. 4) Additionally, D6D and SCD mRNA level in the brain of transgenic fish were down-regulated 14.9-fold and 1.4-fold ($P<0.05$), respectively, compared to non-transgenic fish. Fatty acid (FA) in the muscle of common carp fed the commercial diet had a higher level of EPA, 1.18-fold ($P<0.05$), docosapentaenoic acid (DPA) was 1.11-fold ($P<0.05$) and that of total n-3 was 1.05-fold ($P<0.05$) than in the muscle

of fish fed the formulated diet. The transgenic common carp fed the commercial diet had a 1.07-fold EPA, 1.12-fold DPA, 1.07-fold DHA and a 1.07-fold higher observed total omega-3 fatty acid level than non-transgenic common carp, although these trends were not statistically different ($P<0.05$). However, this observed trend was not present in fish fed the formulated diet. The results demonstrate that the fatty acid metabolic pathway in fish can be modified by the transgenic technology and diet. The long-term goal is to produce common carp that transmit the Masou Salmon D5D transgene to future generations and optimize the transgene construct and feed formula, leading to commercial application to increase the production of food fish, with elevated omega-3 fatty acids reducing the pressure on natural populations of fish containing these valuable fatty acid profiles and adding value to aquaculture products.

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List of Abbreviations and Acronyms

PUFA Polyunsaturated Fatty Acid

LC-PUFA Long Chain- Polyunsaturated Fatty Acid

HUFA Highly Unsaturated Fatty Acid

qRT-PCR Quantitative reverse-transcriptase PCR

FA Fatty Acid

EFA Essential Fatty Acid

EPA Eicosapentaenoic Acid

DHA Docosahexaenoic Acid

OA Oleic Acid

LA Linoleic Acid

ALA α-Linolenic Acid

GLA γ-Linolenic Acid

OTA Octadecatetraenoic Acid

DGLA Epoxyeicosatrienoic Acid

ETA Eicosatetraenoic Acid

AA Arachidonic Acid

SCD Stearoyl-CoA Desaturase

D6D	Δ6-desaturase
D5D	Δ5-desaturase
FAME	Fatty Acid Methyl Ester
Ct Values	Crossing-point Values
SRE	Sterol Regulatory Element
SREBP	Sterol Regulatory Element Binding Protein
PPARα	Peroxisome Proliferator Activated Receptor-α
FO	Fish Oil
VO	Vegetable Oil
CPE	Carp Pituitary Extract
BW	Body Weight
SD	Standard Deviation
CV	Coefficient of Variation

1. Introduction

1.1 Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFAs) refer to long chain fatty acid (C ≥ 16) that contains two or more than two double bonds. PUFAs are predominantly a combination of omega 3 (n-3) and omega 6 (n-6) fatty acids. Omega-3 fatty acids are PUFAs with a double bond starting after the third carbon atom from the end of the carbon chain, and the primary n-3 fatty acids include eicosapentaenoic acid (EPA, C20:5n-3), docosahexaenoic acid (DHA, C22:6n-3), docosapentaenoic acid (DPA, C22:5n-3) and α-linolenic acid. Omega-6 fatty acids are PUFAs with a double bond starting after the sixth carbon atom from the end of the carbon chain and mainly include linoleic acid (LA, 18:2(n-6)) and arachidonic acid (AA, 20:4(n-6) (Yaqoob, 2003). Development of food resources with PUFAs is becoming a field of interest in functional food studies. The metabolism of the omega-3 and omega-6 fatty acids is competitive because both pathways use the same set of enzymes (Simopoulos, 2002). AA, ALA, EPA and DHA cannot be synthesized de novo in mammals and must therefore be obtained from the diet.

1.2 Research Advances on Fatty Acid Desaturases

Fatty acid desaturases are pivotal enzymes for the biosynthesis of PUFAs as they add double bonds into fatty acyl chains at specific sites (Meesapyodsuk et al., 2007). They are present in all groups of organisms and play a key role in the maintenance of the proper structure and functioning of biological membranes. There are three types of fatty acid desaturases: acyl-CoA, acyl-ACP, and acyl-lipid desaturases. According to cofactor requirements and localization they could be classified into two major groups: the soluble and the membrane-bound desaturases. The desaturases possess three conserved histidine tracks, which are assumed to constitute the Fe-binding active centers of the enzymes. Each type of desaturase owns specific consensus protein sequence motifs (Murata and Wada, 1995).

The ability of tissues or organs to synthesize C20 and C22 polyunsaturated fatty acids from C18 fatty acids depends on a complex of desaturases and elongase enzymes. Three types of desaturases operate in animals based on their desaturation site in the fatty acids: Δ9, Δ6 and Δ5, respectively. Stearoyl-CoA desaturase （SCD, Δ9 – desaturase）is a key rate- limiting enzyme involved in the biosynthesis of PUFAs, which affects cellular membrane fluidity, permeability and functionality (Ntambi, 1999). SCD introduces a single bond in the Δ9 position into saturated fatty acyl-CoAs (C18:0) which is the key step in the biosynthesis of monounsaturated fatty acids. This oxidative reaction involves NADPH, cytochrome b5 reductase, and oxygen through the electron transfer chain (Ntambi and Miyazaki, 2004). The product of these reactions is oleic acid (C18:1n9). Metabolism of PUFAs in fish start from Oleic acid

(C18:1n9), and the linoleic acid (C18:2n6, LA) and α-linolenic acid (C18:3n3, ALA) can be synthesized by Δ12-desaturase and Δ15-desaturase.

A proper balance among C18 fatty acids in the feed is necessary to ensure that optimal quantities of the essential C20 and C22 polyunsaturated fatty acids of the omega-6 and omega-3 families will be synthesized. Δ6-desaturase (D6D) is the most important enzyme in these syntheses. Theoretically, C18:3 n-3 is converted to DHA (C22:6 n-3) by a pathway that combines the sequential action of Δ6- and Δ5-desaturase with chain-elongation reactions. Δ6- and Δ5-desaturase are membrane-bound desaturases and fatty acid metabolic enzymes, which behave as important factors in EPA and DHA biosynthesis (Simopoulos, 2002). They allow the introduction of a double bond to different positions on the fatty acids carbon chain. D6D uses LA and ALA as substrates to produce γ-linolenic (C18:3n-6, GLA) and octadecatetraenoic acid (C18:4n-3, OTA). GLA and OTA are converted by elongase to produce epoxyeicosatrienoic acids (C20:3n-6, DGLA) and eicosatetraenoic acid (C20:4n-3, ETA). Δ5-desaturase (D5D) uses DGLA and ETA as substrates to produce arachidonic acid (C20:4n-6, AA) and eicosapentaenoic acid (C20: 5n-3, EPA). DHA is synthesized from EPA by fatty acid chain elongation to C24:5n-3 and double bond insertion by D6D to C24:6n-3, which is finally oxidized by peroxisomes β-oxidation to docosahexaenoic acid (C22:6n-3, DHA) (Nakamura and Nara, 2004a) (Fig 1).

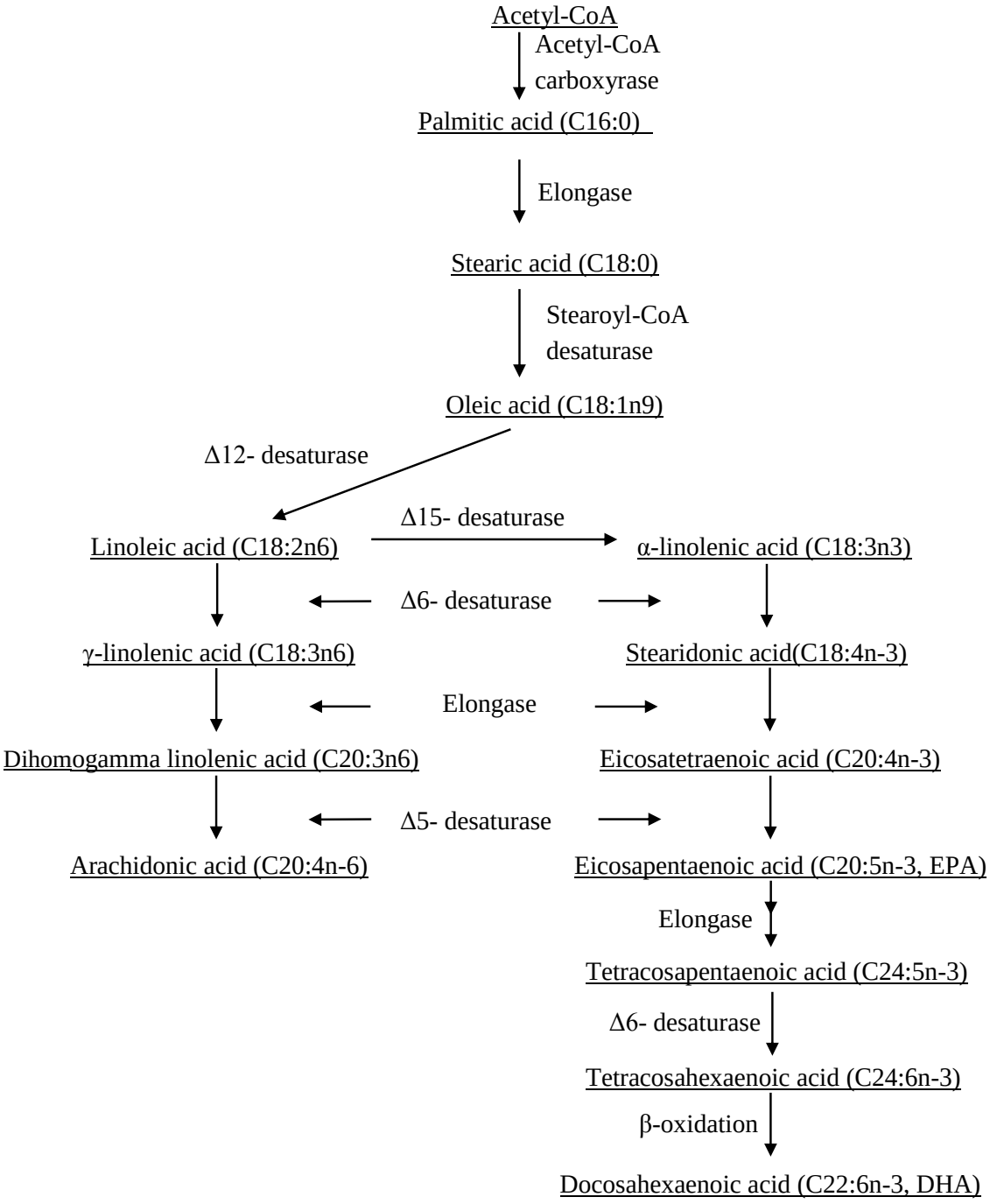


Figure 1 PUFA biosynthesis pathway in vertebrates

1.3 Omega-3 fatty acid content in freshwater and marine fish species

Freshwater fish, marine fish and anadromous fish have similar levels of omega 3 fatty acids (Purdue University, 2013). Coldwater fish tend to have higher levels than warmwater species. The omega 3 fatty acid levels are quite variable among different species of freshwater and marine species, but is less variable in anadromous species (Table 1). Common carp have a value of 0.6g/100g edible fish tissue of n-3 fatty acids, which is on the low side of the range for fish.

Table 1 Omega-3 fatty acid content in fish

	Species	Omega-3 fatty acid level (g/100g edible fish tissue)		
Type of fish	N	Mean (SD)	CV (%)	Range
Freshwater	15	1.5 (1.2)	80.0	0.1-4.6
Marine	31	1.0 (0.8)	80.0	0.2-2.6
Anadromous	7	1.1 (0.3)	27.3	0.6-1.5

N: number of fish species; SD: standard deviation; CV: coefficient of variation

In Atlantic salmon, low dietary levels of C18:3n-3 result in a moderate increase in the percentage of DHA in the liver (Tocher et al., 2002). However, higher dietary doses of C18:3n-3 mainly resulted in accumulation of EPA, and did not result in a further increase in the percentage of DHA which is due to the competition between C18 and C24 for D6D (Ruyter et al., 2000). Marine fish are presumed to have a lower capacity than freshwater fish for conversion of 18C fatty acids into 20-22C

unsaturated fatty acids, which can be explained by the natural selection and evolution. The demands for C_{20} and C_{22} PUFAs in marine fish are accomplished by feeding upon other marine fish which contains these fatty acids in their fish oil. Consequently, many marine fish have poor ability to synthesize C_{20} and C_{22} PUFAs synthesis had been formed for certain marine fish. For example, turbot, lacks C_{18} to C_{20} elongase enzyme resulting in deficiency in the desaturation/elongation fatty acid pathway (Ghioni et al., 1999). There is a low level of Δ5 desaturase activity and desaturation of C20:4n-6 (arachidonic acid, AA) and EPA in gilthead sea bream (Tocher and Ghioni, 1999). In many marine fish such as cod, sea bass, etc., the functional activity and gene expression of D6D is barely detectable (Tocher et al., 2006a, Gonzalez-Rovira et al., 2009). On the one hand, it could be due to a gene loss, gene function loss or different modes of gene regulation corresponding to an adaptation to the omega-3 HUFA-rich marine food web, as also suggested for terrestrial carnivores (Sargent et al., 2003).

1.4 Dietary Effect of PUFAs on Animal Health

PUFAs are necessary for important biological functions of humans, such as regulating lipid metabolism, stimulating growth development, anti-cancer properties, anti- aging properties, immunoregulation, promoting cardiovascular health, and aiding in weight loss (Garg et al., 2006). PUFAs in the diet also help to elevate serum peroxides and depleted antioxidant reserves, and lower plasma triglycerides and help in curing cardiovascular disease (Calder and Yaqoob, 2009). The detailed working

mechanism of polyunsaturated fatty acid in reducing the risk of cardiovascular disease is not yet clear.

Bang et al. (1980) demonstrated that the rarity of ischemic heart disease in Eskimos could partly be explained by the antithrombotic effect of the long-chained PUFAs, especially EPA prevalent in diets rich in marine oils. Several mechanisms have been suggested to explain the anti-arrhythmic actions of PUFAs (Hartog et al., 1986, Kinsella et al., 1990, Nair et al., 1997). For example, higher neutral lipids and phospholipids including AA (C20:4n-6) and DHA (C22:6n-3) were measured in the body of rainbow trout, *Oncorhyncus mykiss,* fingerling fed diets in which the concentration of LA (C18:2n-6) and LNA (C18:3n-3) were raised compared to fish fed diets where LA and LNA were lower (Castell et al., 1972). Kinsella et al. (1990) gave insight into the mechanism of dietary PUFA on incurring the cardiovascular disease. PUFA suppress plasma lipids by inhibiting lipoxygenase, cyclooxygenase, hepatic triglycerides, eicosanoid synthesis, eicosanoid metabolism in macrophages, platelets, and monocytes. These group effects account for the obvious protective nature of PUFA intake, reducing the risk of fatal heart disease. Oudart et al. (1997) designed an experiment in which rats were fed for four weeks a diet without omega-3 PUFAs and a diet with mixture of EPA and DHA. Then the fat composition, GDP binding and uncoupling protein content were measured in the rats. The omega-3 PUFAs limited the development of white fat pads and increased the content of brown adipose tissue. White fat pads were used to store energy that caused obesity while the brown adipose tissues were capable of thermogenesis. Omega-3 PUFAs induced a

marked stimulation of brown adipose tissue thermogenic activity that helps to prevent obesity.

Tian et al. (1996) studied poultry and found that fatty acid synthetase (FAS) has a high positive correlation with the level of abdominal lipids. They noted that appropriate regulation of FAS would be an effective way to control body fat of animals. Clarke et al. (1990) demonstrated that PUFAs decreased mRNA abundance for fatty acid synthases (FAS) by 70%- 90%. PUFAs played an important role on obesity prevention by inhibiting FAS gene expression.

PUFAs help promote ovarian maturation. Wen et al. (2001) fed Chinese mitten-handed crab (*Eriocheir sinensis*) with PUFA enriched diets, and the crabs with PUFA enriched diets had a higher fecundity and egg hatching rate than control group. Koueta et al. (2002) reared juvenile cuttlefish and found that the groups fed a natural diet enriched in PUFAs showed faster growth and exhibited a higher rate of survival than the control group. Copeman et al. (2002) studied the role of DHA, EPA and AA on early growth, survival, lipid composition and pigmentation of yellowtail flounder. There was a strong positive relationship between the DHA/EPA ratio in the diet and larval size and survival. Asturiano et al. (2001) suggested that PUFAs can improve the survival rate of embryos of sea bass.

In early human development, especially in neural and retinal development omega-3 fatty acid is a big concern for most scientists and documented to be important (Dubnov-Raz et al., 2007, Furuhjelm et al., 2007, Innis, 2008, Muhlhausler et al., 2011, Klemens et al., 2012, Sable et al., 2012). The most important omega-3 fatty

acid for early human development is docosahexaenoic acid (DHA) which is now recognized as a physiologically essential nutrient for neuronal functioning of the brain and visual acuity for the retina (SanGiovanni and Chew, 2005). Omega-3 fatty acid deficiency can result in neurological and behavioral disorders, including schizophrenia, Alzheimer's disease, depression, and hyperactivity (Nettleton, 2005). PUFAs improve health, intelligence, and immunity in animals.

1.5 Dietary Regulation of Desaturases

The Δ5-desaturase (D5D), Δ6-desaturase (D6D) and stearoyl-CoA desaturase (SCD) gene expression and enzymatic activity were all modulated by nutritional composition of diets (Zheng et al., 2005a, Zheng et al., 2005b). PUFA and HUFA are high in fish oil (FO) and low in vegetable oil. Consumption of dietary PUFA and HUFA could reduce the D6D and SCD gene expression. For example, higher D6D mRNA levels were observed in the sea bass larvae fed a low HUFA diet (0.5% or 0.7% EPA+DHA) than a high HUFA diet (1.7% or 3.7% EPA+DHA) (Vagner et al., 2009a). Similarly, González-Rovira et al (2009) reported that the D6D mRNA level in the liver of sea bass fed linseed and rapeseed oil was higher than fish fed FO. The regulation of desaturases by PUFA is related to two transcription factors, sterol regulatory element binding protein (SREBP) and peroxisome proliferator activated receptor-α (PPARα) (Nakamura and Nara, 2004b). PUFA suppresses the desaturases by binding to sterol regulatory element (SRE), which promotes the expression of

many genes such as SCD, fatty acid desaturase, D6D and elongase. PPARα is responsible for the FA oxidation (Nakamura and Nara, 2004b).

1.6 Transgenic Technology

Gene- transfer research of fish, including goldfish (Zhu et al., 1985), rainbow trout (Chourrout et al., 1986), channel catfish (Dunham et al., 1987) and Nile tilapia (Brem et al., 1988) was initiated in the mid- 1980s. Gene transfer technology has the potential for introducing significant new sources of variation into the germline of a species by introduction of new or extra copies of cloned genes. Most efforts in transgenic fish important for aquaculture have been devoted to growth enhancement, improvement of cold- and disease-resistance (Fletcher and Davies, 1991, Du et al., 1992).

Du et al. (1992) developed an "all fish" growth hormone (GH) chimeric gene construct by using an antifreeze protein gene (AFP) promoter from ocean pout linked to a chinook salmon GH cDNA clone. They transferred the "all fish" growth hormone (GH) chimeric gene into the Atlantic salmon and results showed dramatic increases in their growth rate. At one year old, the average increase of the transgenic fish was 2 to 6 fold and the largest transgenic fish was 13 times that of the average non-transgenic control.

Recently, Lee et al. (2009) observed that knockdown of myostatin zebrafish with an antisense RNA- expressing construct had dramatic positive effect on the myostain

gene expression, myostain protein levels, myogenic regulatory factors and larger size muscle fiber in transgenic fish compared to non- transgenic fish. Dunham et al. (2002) demonstrated that the transgenic F1 (transgenic fish offspring) individuals containing the cecropin B gene from the moth *Hyalophora cecropia* had higher survival rate and disease resistance when challenged with pathogenic bacteria compared to non-transgenic fish. Yazawa et al. (2006) used a chicken lysozyme gene construct to make transgenic zebrafish, and reported that the F2 transgenic fish challenged with both *Flavobacterium columnare* and *Edwardsiella tarda* exhibited higher survival rates compared to non-transgenic fish. Currently, reporter genes, especially green fluorescent protein (GFP) gene are widely used to study various biological phenomena (Li et al., 2009). Lee et al. (2007) indicated that the GFP transgenic zebrafish played an important role in understanding the mechanism of Alzheimer's disease. Chen et al. (2010) used a GFP construct driven by the vitellogenin promoter to generate transgenic zebrafish. Green fluorescence was observed in the fish liver when the transgenic fish were exposed to 17-α-ethynylestradiol (EE_2) since the EE_2 could bind to its receptor vitellogenin. The results showed that the transgenic fish could be a promising environmental monitoring. Transgenic technology can also use help to enhance performance for animals. The mature tilapia insulin-like growth factors (IFGs) proteins were produced by using the zebrafish eggs as bioreactors, and the results indicated that the zebrafish eggs can be used as bioreactors to produce the biological recombinant proteins (Hu et al., 2011).

As aquaculture production grows, it becomes an increasingly necessary to apply modern biotechnologies to meet people's growing needs for aquaculture products in both quality and quantity. Many countries, including the US (Dunham, 2009), Canada(Lang and Beatty, 2007), China (Liu et al., 2001), Cuba (Ojalvo et al., 2003), India (Dutta and Pan, 2002), Korea (Joo et al., 2012), Japan (Yazawa et al., 2005), have active research programs in the area of genetically engineered organisms. Many gene transfer methods have been utilized for fish transgenesis, such as microinjection, electroporation, gene gun bombardment, sperm-mediated, direct mixing, liposomes-mediated, and gonad direct injection (Dunham and Liu, 2006). Transgenic technology and application are quite effective in fish now.

The isolation of genes responsible for desirable traits, and their transfer as fusion genes could provide genetic enhancement that surpasses traditional selection and breeding methods. Additionally, new traits not existing in a genome can be transferred to different species, enabling the production of new phenotypes (Fletcher and Davies, 1991). Currently research on genetically modified fish is mainly focused on the growth hormone gene, disease-resistant protein gene and transgenic sterilization (Dunham et al., 1987, Dunham et al., 2002, Wu et al., 2003, Dunham, 2011).

1.7 Desaturase Genes Cloning and Gene Transfer Research

Over the past few years, a number of fatty acid desaturases and their genes had been isolated and sequenced from different organisms, including bacteria, fungi, plants and animals. In the animal kingdom, two main families of desaturases,

corresponding to SCD and to D5D and D6D, have been defined (Marquardt, 2000). Enzymes from both family present three typical histidine boxes and two membrane-spanning domains, but D5D and D6D contain an additional N-terminal cytochrome *b*-like domain, absent in SCD. Desaturase sequences of both families are available in human, rats, mice, sheep, fish and insects (Seiliez et al., 2001). In fish, the desaturase sequences were obtained from zebrafish (GenBank accession numbers AF309556), carp (Tiku et al., 1996), salmon (GenBank accession numbers AB070444), rainbow trout (Seiliez et al., 2001), milkfish (Hsieh et al., 2001) and gilthead seabream (Seiliez et al., 2003). Additionally, an EST sequenced from the liver of channel catfish was similar to SCD gene in other species (GenBank accession numbers BM438315). Analyzing these sequences and the gene expressions in fish are necessary for understanding biochemical mechanisms and processes of PUFA conversion, which has strong implications with the field of fish nutrition.

Gene transfer was used in several plants, such as tobacco (Hamada et al., 1998), rapeseed (Knutzon et al., 1992), to increase linolenic acid level and modulate the rate of different fatty acids. The approach in rapeseed was to alter the enzyme systems for fatty acid desaturation and elongation to make omega-3 fatty acids. Reddy et al (1996) cloned a cyanobacterial delta-6 desaturase gene, and expression of this gene in transgenic tobacco resulted in gamma- linolenic acid accumulation.

Zhang et al (2009) transferred Δ12 fatty acid desaturase gene from maize into *Saccharomyces cerevisize* and the results showed that the transferred yeast cells expressing FAD2 had a linoleic acid content of 1.54% of total fatty acid content,

which was not present in control yeast cells. The maize Δ12 fatty acid desaturase gene was functional in yeast.

In the case of fish, Alimuddin et al (2005, 2007) transferred masou salmon ∆6-desaturase-like and ∆5-desaturase-like gene in zebrafish, resulting in increased EPA and DHA compared to the non-transgenic fish. Cai et al. (2012) studied the biological effects of the expression of fatty acid desaturase gene in vivo in the rabbit and explored the relationship between unsaturated fatty acids and immune system of rabbit. The transferred desaturase gene could be effectively expressed in experimental rabbits and improved the content of PUFAs that can significantly enhance in vivo and acquired immune function.

With the development of biotechnology, especially the application of genetic engineering, it may be possible to obtain functional fatty acids through genetic manipulation of fatty acid desaturase to control the composition of PUFAs in organisms. Compared to the marine fish, freshwater fish contains high level proportions of C18 PUFA (Purdue University 2013). One objective of the current study was to utilize diet regulation and transgenic technology to elevate the omega-3 fatty acid in common carp. Specific objectives were to transfer the Masou salmon Δ5-desaturase-like gene to common carp to determine its effect on transcription level of endogenous gene Δ6-desaturase-like gene and stearoyl-CoA desaturase and to determine the effects of this transgene on fatty acid composition.

2. Materials and Methods

2.1 Transgene Construction

A backbone construct (NCBI accession#: AF170915.1) was modified as follows. The sequence of green-fluorescence protein was excised from the backbone and substituted with a 1.4-kb Oncorhynchus Masou delta5-desaturase-like gene (D5D, Accession ID: EU098126.1). The 1.4-kb D5D was driven by the common carp β-actin promoter. The synthetic, desaturase construct (8.3 kb) was built at GenScript (USA Inc., Piscataway, NJ 08854, USA).

2.2 Plasmid DNA preparations

Plasmid was transformed with One Shot® Top10 chemically competent *E.coli* cell (cat#: C4040-10, Invitrogen, Corp.,NY, USA) following the procedures recommended by the protocol. About 100 to 200 µl transformation mix was used to plate on LB agar (10.0 g tryptone, 5.0 g yeast extract, 5.0 g NaCl, 15.0 g agar in 1.0 L solution) plates. A single colony was picked from each plate to prepared 300ml LB medium (10.0 g tryptone, 5.0 g yeast extract, 5.0 g NaCl in 1.0L solution) the culture grown overnigh at 37°C with vigorous shaking (200-300rpm) to A_{600} value of 1.2-1.5. Plasmid DNA extraction was conducted using a mini-prep plasmid kit.

(Qiagen mini-prep, CA, USA). The harvested cells from the 300ml LB medium were transferred to a 250ml centrifuge bottle and the bacteria cells were pelleted by centrifugation for 10min at 3,000×g at room temperature. The bacteria cells were resuspended in 10ml buffer A which containing 0.1 mg/ml RNase A. Then 10ml lysis buffer B was added to the bottle and inverted gently 4-6 times to mix. Then 15ml neutralization buffer C1 was added to the bottle and inverted immediately but gently 4-6 times. The lysate was centrifuged for 10 min at maximum speed, 15,000-18,000×g, at 4°C. Then the supernatant was transferred to the DNA binding column unit and the column centrifuged for 5 min at 3,000×g at room temperature. Then, the DNA binding column was removed from the unit, the liquid from the collection tube was discarded and the DNA binding column unit was reassembled. Then, 20ml of 70% EtOH was added to the DNA binding column unit to wash the plasmid and the unit centrifuged at 3,000×g for 5 min at room temperature. After this step, the DNA binding column was transferred to a new 50ml centrifuge tube and 1ml of preheated sterilized water was added to the center of the DNA binding column and left at room temperature for 1min. The plasmid DNA was then eluted by centrifuging the unit for 5 min at 3,000×g at room temperature.

Plasmids were linearized with SfiI (20,000 units/ml, New England Biolabs, MA, USA) following the product's protocol with little modification. For plasmid digestion, a mixed 50.0 µl reaction solution containing 1.0 µg of plasmid DNA, NEBuffer 4(10×), BSA (100×) was utilized and the volume adjusted using water to 50.0 µl.

DNA agarose gel electrophoresis was used to analyze the plasmid. A maxi-prep plasmid DNA extraction was performed using Qiagen maxi-prep kit once the size of the construct was confirmed. A phenol-chloroform-ethanol method was used to inactivate SfiI enzyme and purify the linearized plasmid DNA. The specific steps were as follows: 500µl of plasmid DNA (finished enzyme digestion) was prepared in a 1.5ml tube. Then 500µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the solution and vortexed 30s. The solution was centrifuge in a microfuge at full speed (13,000-16,000rpm) for 6 min to separate the phases. The supernatant was transferred to a new tube (avoiding the white pellet in the interphase). 400µl of chloroform/isoamyl alcohol (24:1) was added to the solution and vortexed for 5s. The solution was centrifuged at full speed for 2 min. The upper phase was transferred into a new 1.5ml tube and 30µl 3M NaOAC + 600µl 100% EtOH (pre-chilled, keep in -20°C) was added to the solution and inverted for 30 times or more. The solution was stored at -20°C for 2 hours to allow the precipitation of plasmid DNA followed by centrifugation at 14,000rpm for 15 min and then decant the supernatant was decant as much as possible. One ml pre-chilled 70% EtOH was added and the DNA pellet was washed 2 times. Finally the sterilized water was added to washed and dry DNA pellet and kept in -20°C or for later use. The quantity of DNA was measured using a UV-spectrophotometer.

The plasmid was prepared separately in two tubes for the purpose of double electroporation. One was diluted in the 2.0 ml (0.9%) saline with a concentration of

50 µg/ml of the transgene. The other for the second electroporation was prepared in 9.0 ml TE buffer (5 mM Tris-HCl, 0.5 M EDTA, pH=8.0) with a concentration 50 µg/ml.

2.3 Brood stock spawning

Sexually mature male and female common carp were harvested from the Fisheries Genetics Unit, EW Shell Research Center (Auburn University, AL) and acclimated to 25-27 °C. Males and females were separated with females in the inlet side and males in the drainage side. Sperm was collected from 3 common carp males, diluted with 9 ppt saline (container 0.9% sodium chloride, injection, USP, MA, USA), then refrigerated until use. Three females were artificially induced to ovulate with carp pituitary extract (CPE) at 0.4 mg/kg body weight (BW) for the priming dose and injection 3.6 mg/kg BW as the resolving dose 9 hr later.

2.4 Fertilization, electroporation and incubation

Electroporation was performed with the Baekon 2000 macromolecule transfer system (Baekon, Inc., Saratoga, California, USA). Parameters were 6 kV, 2^7 pulses, 0.8 sec burst, 4 cycles, 160 µsec (Powers et al., 1992). One or two drops of sperm were placed in the desaturase plasmid solution (1.0 ml) in saline and mixed. Sperm was kept in this solution a minimum of 5 minutes before use. Then DNA/ sperm solution was poured into a 7 ml petri dish and completely filled with fresh water.

Then the sperm was electroporated with the Baekon 2000 macromolecule transfer system. Then the common carp eggs were fertilized with the electroporated sperm. The fertilized embryos were loaded into the petri dishes and completely covered with the desaturase plasmid solution in TE buffer after 10 minutes. The eggs remained in the DNA for 10 minutes. Then the fertilized eggs were electroporated. Embryos were then moved into 8.0 L tubs with 5.0 L Holtfreter's solution, and incubated statically. The embryos were gently agitated with compressed air delivered through airstones. Dead embryos were removed daily before changing Holtfreter's solution.

2.5 Experimental feeds, Diet Treatment and Sample collection

Embryos were cultured for three days to larvae in tubs at 26°C. Larvae were fed artemia, then Aquamax Fry Starter100 (Cat#: 000-5553, Aquamax, MO, USA) once daily. Fry were transferred into a recirculating system and randomly separated into three tanks at forty days. Then, fingerlings were fed Aquamax fingerling starter 300 (Cat#: 000-5555, Aquamax, MO, USA), containing 50% crude protein, 16% crude fat, 3.0% crude fiber, 0.35% sodium, 2.2% calcium, 1.5% phosphorus, vitamin A, C, D, and E (Table 2).

Table 2 Percent protein, fiber, fat, phosphorous and vitamin content in a commercial and formulated diet with Masou salmon Δ5-desaturase transgenic and non-transgenic common carp, *Cyprinus carpio*.

	Commercial Diet	Formulated Diet
Crude protein (%)	50	36
Crude fiber (%)	3	
Crude fat (%)	16	6
Phosphorus (P) (%)	1.5	0.74
Vitamin A (IU/kg)	9,000	2,200
Vitamin D (IU/kg)	2,400	1,100
Vitamin E (IU/kg)	110	30
Vitamin C (mg/kg)	200	250

Eight months later, 60 juvenile fish were randomly assigned to two groups. Thirty fish were fed a commercial diet and thirty with a formulated diet for one month. The Formulated diet was prepared at the Nutrition Laboratory (School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University, Auburn, AL, USA). The formulation contained 36% protein using soybean meal solvent extracted as the primary protein source and canola oil as the source of essential fatty acids (EFAs) (Tables 2, 3).

After one month feeding of the diets, all 60 juvenile common carp were sacrificed and each fish individually weighed and body length measured. Four different tissues from each fish, including brain, muscle, liver, and gonad, were collected. These samples were immediately frozen in liquid nitrogen and stored at -80°C until DNA and RNA extraction.

Table 3 Ingredient composition (g 100g-1 of feed) of a formulated diet fed to common carp, *Cyprinus carpio*, electroporated with Oncorhynchus Masou Δ5-desaturase-like gene and a normal control.

Ingredient	Weight (g)
Corn gluten meal[a]	8
Corn Starch[a]	1.9
Soybean meal solvent extracted[b]	35.5
Canola oil[c]	3.6
Whole wheat[d]	38.8
Choline cloride[d]	0.2
Trace minerial premix[e]	0.5
Vitamin premix[f]	0.8
Stay C 250mg/kg (25%)[g]	0.1
CaP- diabsic[h]	0.5
Lecethin(deoiled 53% lipid)[i]	0.5
Lysto[j]	8
Methionine[j]	0.15
L-lysine[j]	0.45
Filler (alpha cell/nonnutritive filler)[j]	1
Total	100

[a] Grain Processing Corporation, Muscatine, IA, USA.

[b] Faithway Feed Co., Guntersville, AL, USA.

[c] ConAgra Food Co., Omaha, NE, USA.

[d] MP Biochemicals Inc., Solon, OH, USA.

[e] Trace mineral premix (g/100 g): cobalt chloride 0.004, cupric sulphate pentahydrate 0.250, ferrous sulfate 4.0, magnesium sulfate heptahydrate 28.398, manganous sulphate monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulfate heptahydrate 13.193, filler 53.428.

[f] Vitamin premix (g/kg): thiamin HCl 0.5, riboflavin 3.0, pyridoxine HCl 1.0, DL Ca-pantothenate 5.0, nicotinic acid 5.0.

[g] Stay C®, (L-ascorbyl-2-polyphosphate), Roche Vitamins Inc., Parsippany, NJ, USA.

[h] Fisher Scientific, Fair Lawn, NJ, USA.

[i] Solae Company, St. Louis, MO, USA.

[j] Sigma-Aldrich Co., St. Louis, MO, USA.

2.6 Transgene identification and gene quantification

Genomic DNA was extracted using proteinase K digestion followed by protein precipitation and ethanol precipitation utilizing the protocol described (Kurita, et al., 2004). A total of 600µl cell lysis solution (100mM NaCl, 10mM Tris, 25mM EDTA, 0.5%SDS) and 3µl Proteinase K were added to each sample and vortexed for 20s. The mixed solution was incubated in a water bath at 55°C for 3 hours until the tissue was dissolved. After incubation, samples were cooled to room temperature. 200µl protein precipitation solution was added to each sample followed by vigourous vortexing for 20s and then samples were placed on ice for 15 min. The sample was then centrifuged at 15,000 rpm for 5-10 min at room temperature. The supernatant was poured into a labeled 1.5ml tube containing 600µl isopropanol and mixed by inverting gently 50 times. The mixed solution was centrifuged at 15,000 rpm for 6 min at room temperature. Then the supernatant was poured off and the small white pellet with DNA remained. 600µl of 70% EtOH was added to wash the DNA pellet and then centrifuged at 15,000 rpm for 2 minutes at room temperature. After this step, the EtOH was carefully poured off and the tube was gently inverted on paper towels to air dry the pellet for 5-10 min. Then 50-100µl sterilized water was added to dissolve the DNA pellet. The DNA was run in a 1.2% agarose gel with Ethidium Bromide (1-2µl of EtBr/100ml gel) and the DNA measured with a spectrophotometer. PCR was performed on each tissue to identify which tissues contained the transgene. The amplification procedure was as follows: initial denaturation for 4 min at 94 °C,

followed by 35 cycles of 94°C for 30s, 58°C for 30s and 72°C for 70s and the final elongation for 10 min at 72°C.

All transgenic and control samples from various tissues were ground into powder in the presence of liquid nitrogen, and approximately 0.3 ml of powder from each sample was used to extract RNA using TRIzol® Reagent (Ambion, cat #: 15596-018, NY, USA). The RNA extraction steps were as follows: The powdered tissue was taken from -80°C and placed on ice. One ml TriZol was added to each tube of sample powder and a syringe was used to mix the solution well, left at room temperature for 5 min. Then 0.2ml of chloroform was added to each tube and vortexed for 5s, and then centrifuged at 12,000 rpm for 15 min at 4°C. After centrifugation, the top aqueous layer was transferred to a new tube, and 0.25ml isopropanol was added to each tube, and mixed by hand at room temperature for 10 min. Then the solution was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was poured off and 1ml 75% EtOH-DEPC was added to each tube to wash the RNA pellet followed by centrifugigation at 7,500 rpm for 5 min at 4°C. Then the EtOH-DEPC was carefully poured off and the tube was gently inverted on paper towels to air dry the RNA pellet for 5-10 min. After this step, 60µl DEPC water was added to each tube. The quality and quantity of RNA was measured using a DNA agarose gel and UV-spectrophotometer. All extracted samples had an A260/280 ratio greater than 1.8, and were diluted to 500 ng/µl.

Table 4 Primer sequence for identifying Masou salmon Δ5-desaturase-like gene and real-time PCR for common carp, *Cyprinus carpi*o.

Gene	GID	Forward Sequence	Reverse Sequence	Size (bp)
D5D	Construct	GCCTCTGCTAACTGGTGGAA	CAGCAAAGCCATGTAGCAAA	903
D6D	AF309557.1	GCACAGTCACAGGCTGAATGGT	GATGCTGGAAGTGGCGATGGTT	151
SCD	U31864.2	ATCCGGACGTCATTGAGAAG	AATACGCCACCCACAGAGAC	171
18S	FJ710827.1	CCTGCGGCTTAATTTGACTC	CCGGAGTCTCGTTCGTTATC	165

Primers were used for screening positive transgenic individual from the population and quantification gene expression using quantitative reverse-transcriptase PCR (qRT-PCR).
D5D: Δ-5-desaturase-like gene from *Oncorhynchus masou*.
D6D and SCD: Δ-6 desaturase-like gene and stearoyl-CoA desaturase gene (SCD) from common carp, *Cyprinus carpio*, respectively.
18s: 18s ribosome mRNA from common carp, *Cyprinus carpio*.

RNA was reverse transcribed into cDNAs by iScript Synthesis Kit (Bio-Rad, USA, cat #: 170-8891). qRT-PCR was performed on a C1000 Thermal Cycler (Bio-Rad, USA) by using SsoFast EvaGreen supermix kit (Bio-Rad, cat: #172-5201, USA,) following the manufacturer's instructions with modification. Ribosome 18s mRNA was used as an internal control. Primers used for transgene D5D detection and relatively quantification of delta-6-desaturase like gene (D6D) and Stearoyl-CoA desaturase (SCD) were listed in table 4.

2.7 Lipid extraction and fatty acid analysis

The muscle from all transgenic fish and 10 non-transgenic fish for each diet group was collected for FA analysis. Total lipids from tissues and feed samples were extracted and individually determined gravimetrically by homogenization in triplicate

aliquots of choloroform/methanol (2:1; v: v) after Folch et al (Folch et al., 1957) with some modifications. Fatty acid methyl ester (FAME) was prepared by transesterification from FA with boron trifluoride and analyzed by using a gas chromatography equipped with flame ionization detector (GC-17A, Shimadzu, Portland, OR, USA). Separation and identification were achieved by OMEGAWAX™ 530 capillary column (30 m × 0.53 mm × 0.5 μm, Supelco, Oslo, Norway) and by the comparison of retention time with the FAME standard (Methyl nonadecanoate≥98%, Sigma-Aldrich Co., St. Louis, MO, USA), respectively. The initial oven temperature was 140°C and was then ramped to 260°C, and held for 5 minutes. The temperatures of injector and detector were set as 260°C and 270°C, respectively. A sample of 2 μL was injected under spiltless mode. FAs were expressed as mg/g wet weight and as percent of the total identified FAME.

2.8 Statistical analysis

Crossing-point (Ct) values were exported into Microsoft Excel sheet from Bio-Rad CRX Manager (Version 1.6.541.1028, 2008). Relative expression analyses was conducted for 4 types of comparisons. Relative gene expressions were expressed as fold change of different tissues (brain, muscle, gonad) compared to liver in non-transgenic tissues. Relative gene expressions were expressed as fold change of different tissues (brain, muscle, gonad) compared to liver in transgenic tissues. Relative gene expressions were expressed as fold change of different tissues (liver,

gonad, brain, muscle) in non-transgenic common carp fed on formulated diet compared to that of fish fed commercial diet. Relative gene expressions were expressed as fold change of different tissues (liver, gonad, brain, and muscle) in transgenic common carp compared to that of non-transgenic samples fed commercial diet. The expression of 18s ribosomal mRNA was used as an internal control. The relative expression ratio of target gene was analyzed for significance test using a randomization test in the REST (Pfaffl et al., 2002) software on the assumption that PCR had 100% efficiencies, and randomization was performed at 2,000 times to capture significance at the level of $P<0.05$. The results were graphed with corresponding standard errors.

Statistical analyses were conducted by using SAS statistical software (version 9.1, SAS Institute Inc., Cary, NC, USA). Analysis of variance in fatty acid composition of different treatments was performed by a one-way ANOVA, followed by Duncan's multiple range test ($p<0.05$). Comparison of transformation rate among tissues was accomplished with Fisher's exact test of independence ($P<0.05$). Body weight gain comparisons were conducted with paired-t-tests ($P<0.05$).

3. Results

3.1 Body weight gain on commercial and formulated diets

The initial body weight of 30 fish fed formulated diet and 30 fish fed commercial diet were 32.3g/fish and 36.0g/fish, respectively. After one month experiment diet treatment the body weight of 30 fish fed formulated diet and 30 fish fed commercial diet were 34.9/fish g and 40.0g/fish, respectively. The increase weight of fish fed formulated diet was 2.6g/fish and of fish fed commercial fish were 4.0g/fish, respectively.

3.2 Total lipid content of experimental diets

Total lipid content of the commercial diet was twice that of the formulated diet (Table 5). The substrates LA (C18:2n-6) and ALA (C18:3n-3) was 4-fold and 5.8-fold in the formulated diet than the commercial diet. The ratio of AA (C18:2n-6)/ ALA (C18:3n-3) in formulated and commercial diet was 4.6 and 6.6, respectively. The EPA, DHA and total n-3 in the commercial diet was 31.3-fold, 26.3-fold and 2.6-fold higher than in the formulated diet, respectively. The content of PUFA in the formulated diet was 2.7-fold higher than the commercial diet, and HUFA in the commercial diet was 16.3-fold than in formulated diet.

Table 5 Fatty acid (FA) composition (mg g-1 diet and % of fatty acid methyl ester, FAME) of a formulated diet and commercial diet1 fed to common carp, *Cyprinus carpio*, electroporated with Masou salmon delta5-desaturase-like gene and a non-transgenic control[1] (N=3 for each diet).

Fatty Acid	Formulated Diet	Commercial Diet
Total lipid (%)	6.29	13.62
mg/g wet weight		
16:0	7.22	11.68
18:0	1.86	2.36
18:1n-9	25.59	6.55
18:2n-6	18.28	4.55
18:3n-3	3.97	0.69
20:4n-6	0.00	0.08
20:5n-3	0.18	5.63
22:5n-3	0.38	1.04
22:6n-3	0.13	3.42
Saturates[2]	15.77	22.84
Monounsaturates[3]	26.95	14.98
PUFA[4]	22.49	8.40
HUFA[5]	0.69	11.23
Total n-3[6]	4.80	12.54
Total n-6[7]	18.30	4.81
FAME %		
16:0	10.95	20.34
18:0	2.83	4.10
18:1n-9	38.83	11.44
18:2n-6	27.75	7.92
18:3n-3	6.02	1.20
20:4n-6	0.00	0.13
20:5n-3	0.28	9.78
22:5n-3	0.53	1.81
22:6n-3	0.20	5.96
Saturates[2]	23.97	39.78
Monounsaturates[3]	40.90	26.11
PUFA[4]	34.13	14.61
HUFA[5]	1.01	19.50
Total n-3[6]	7.23	21.79
Total n-6[7]	27.78	8.38

[1] Values represent averages of triplicates samples. Total lipid was calculated as percent of wet tissue.
[2] Saturates: 14:0, 15:0, 16:0, 18:0, 20:0, 22:0. Internal standard, 19:0, was not considered.
[3] Monounsaturates: 15:1, 16:1 18:1, 20:1.
[4] PUFA: 16:2, 16:3, 18:2, 18:3, 20:2, 20:3, 22:2.
[5] HUFA: 18:4, 20:4, 20:5, 22:4, 22:5, 22:6.
[6] Total n-3: 18:3n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3.
[7] Total n-6: 18:2n-6, 20:3n-6, 20:4n-6.

3.3 Masou salmon D5D transgene distribution in different tissues of common carp

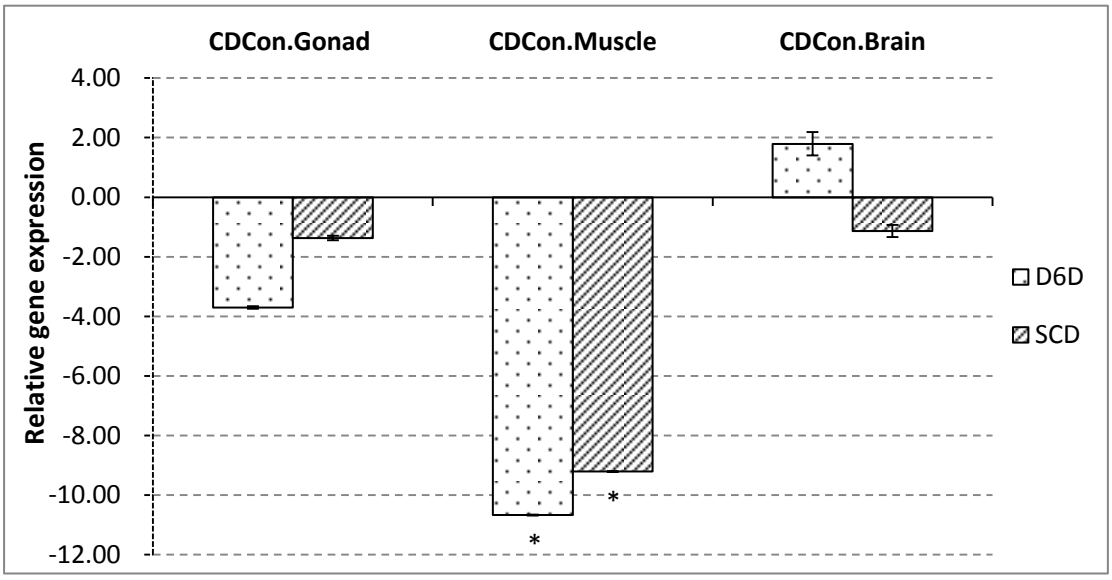
The transgene was randomly distributed in all four tissues screened, liver, muscle, brain and gonad with 8.3%, 21.7%, 10% and 26.7% of individuals transgenic, respectively, for these 4 tissues. In some cases, the transgene was found in 2 tissues (1.7-5.0%), but no fish were detected that were transgenic in 3 tissues. (Table 6)

Table 6 Percentage of transgenic (P1) fish of common carp, *Cyprinus carpio*, for Masou salmon delta5-desaturase-like gene by tissue; liver, muscle, brain and gonad, as determined by PCR analysis. Transfromation rate was different among tissues (P=0.017, Fisher's Multi-treatment Exact Test).

Experiment Diet	Tissue	Number	Transgenic Percentage (%)
Formulated Diet	Liver	3	8.3
Commercial Diet	Liver	2	
Formulated Diet	Muscle	6	21.7
Commercial Diet	Muscle	7	
Formulated Diet	Brain	1	10.0
Commercial Diet	Brain	5	
Formulated Diet	Gonad	6	26.7
Commercial Diet	Gonad	10	
Formulated Diet	Muscle, Liver	1	1.7
Commercial Diet	Muscle, Liver	0	
Formulated Diet	Brain, Liver	1	1.7
Commercial Diet	Brain, Liver	0	
Formulated Diet	Muscle, Gonad	1	5.0
Commercial Diet	Muscle, Gonad	2	
Formulated Diet	Liver, Gonad	0	1.7
Commercial Diet	Liver, Gonad	1	
Formulated Diet	Brain, Gonad	0	1.7
Commercial Diet	Brain, Gonad	1	

3.4 Tissue distribution of D6D and SCD mRNAs in non-transgenic fish fed commercial diet

Expression of D6D and SCD was not different (P>0.05) among liver, brain and gonad of non-transgenic common carp fed a commercial diet (Fig. 2). Expression of D6D and SCD was approximately 10-fold less (P<0.05) in muscle compared to liver.



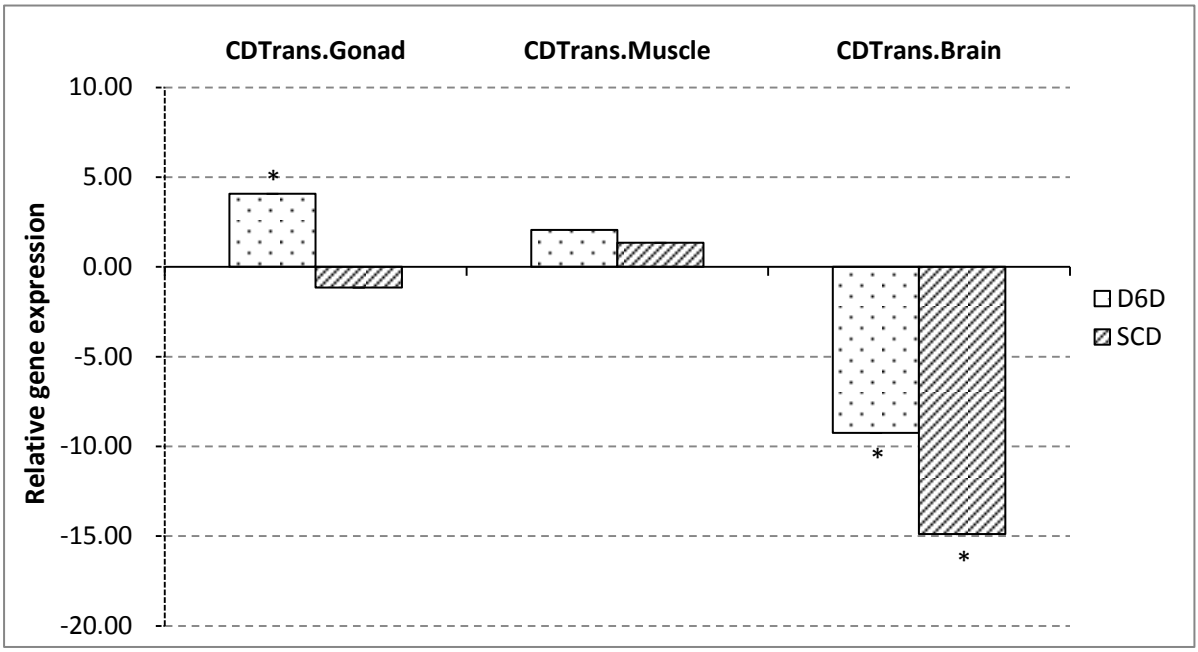
Relative gene expressions were expressed as fold change of different tissues (brain, muscle, gonad) over liver as normalized to the expression of common carp (*C. carpio*) 18s ribosomal mRNA control. Relative fold changes were expressed as mean ± SE. The * on the bar indicated the relative expression between tissue and liver was significantly different from each other at P < 0.05 using Pairwise Fixed Reallocation Randomization Test (PFRR).

CDCon: non-transgenic fish fed commercial diet

Figure 2 Analysis of relative tissue-specific expression of Δ6-desaturase (D6D) and stearoyl-CoA desaturase (SCD) gene in 9-month old non-transgenic common carp (*Cyprinus carpio*) fed a commercial diet for 8-months.

3.5 Tissue distribution of D6D and SCD mRNAs in transgenic fish fed commercial diet

Expression of D6D and SCD in muscle and expression of SCD in gonad was not different (P>0.05) from that of liver in transgenic common carp fed a commercial diet (Fig.3). Expression of D6D was 4.1-fold higher in gonad compared to liver. Expression of D6D and SCD was 9.2-fold and 14.9-fold lower in brain compared to liver, respectively.



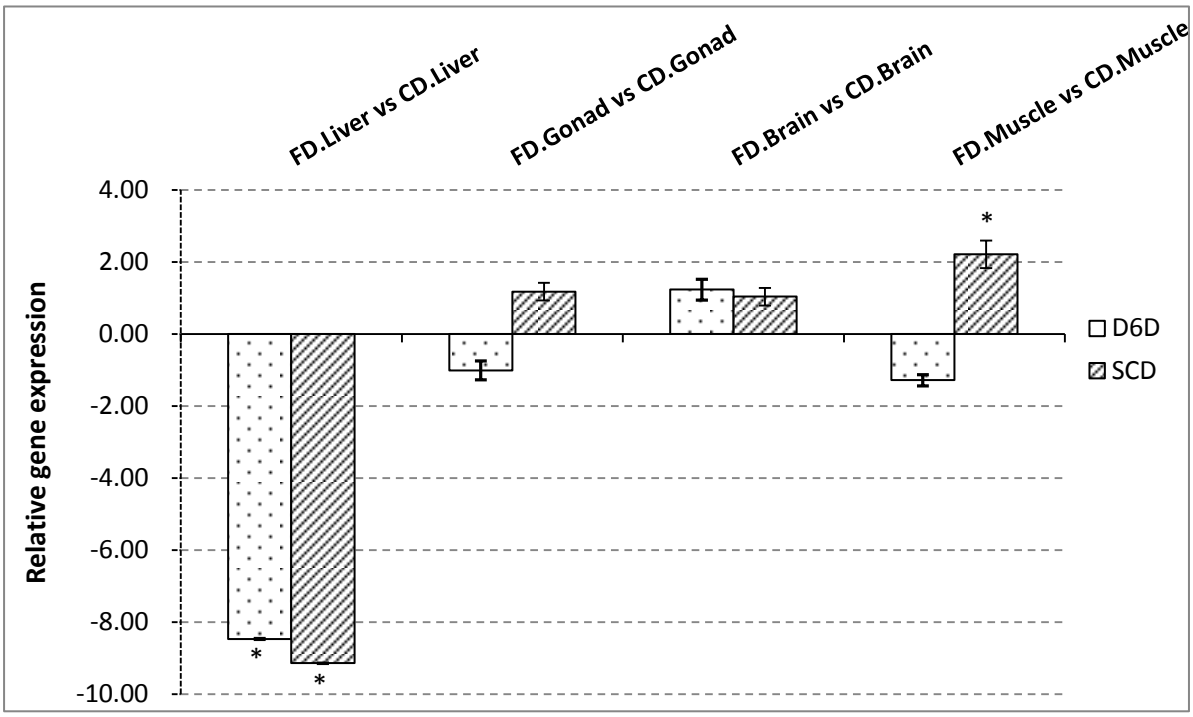
Relative gene expressions were expressed as fold change of different tissues (brain, muscle, gonad) over liver as normalized to the expression of common carp 18s ribosomal mRNA control. Relative fold changes were expressed as mean ± SE. The * on the bar indicated the relative expression between that tissue and liver was significantly different from each other at $P < 0.05$ using the Pairwise Fixed Reallocation Randomization Test (PFRR).

CDTrans: transgenic fish fed commercial diet

Figure 3 Analysis of relative tissue-specific expression of Δ6-desaturase (D6D) and stearoyl-CoA desaturase (SCD) gene in 9-month old transgenic common carp (*Cyprinus carpio*) fed a commercial diet for 8-months.

3.6 Comparasion of D6D and SCD mRNA level in non-transgenic fish treated with two experiments diets

In liver, D6D and SCD transcripts decreased ($P<0.05$) when the diet was changed from commercial diet to formulated diet for non-transgenic common carp (Fig. 4). The D6D mRNA level was 8.5-fold and SCD mRNA level was 9.1-fold higher in fish liver fed commercial diet than that fed formulated diet. SCD transcripts in muscle increased significantly after the diet were changed from commercial diet to formulated diet ($P<0.05$). The SCD mRNA level was 2.2-fold higher in fish muscle fed formulated diet than that fed commercial diet ($P<0.05$). D6D and SCD transcripts in gonad and brain were not different ($P>0.05$) between fish fed formulated diet and commercial diet.



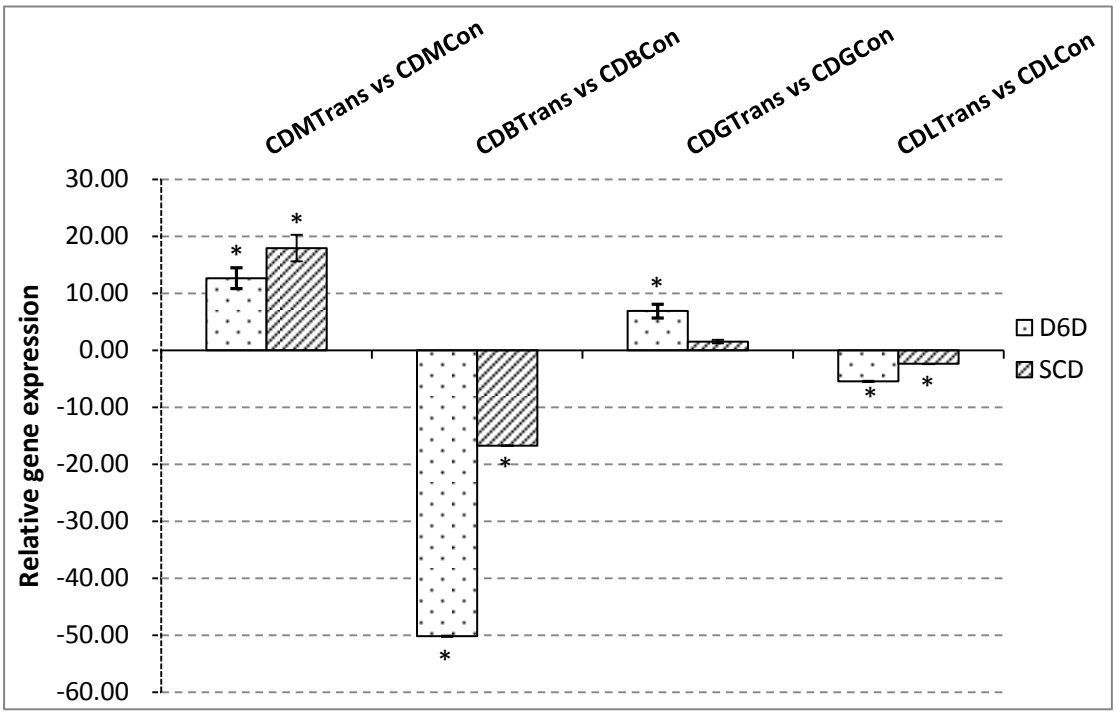
Relative gene expressions were expressed as fold change of different tissues (liver, gonad, brain, muscle) in non-transgenic common carp fed on a formulated diet over that of those fed commercial diet as normalized to the expression of common carp (*C. carpio*) 18s ribosomal mRNA. Relative fold changes were expressed as mean ± SE. The * on the error bar indicated the relative expression between fish fed formulated diet and fish fed commercial diet was significantly different from each other at $P < 0.05$ using Pairwise Fixed Reallocation Randomization Test (PFRR).

FD: formulated diet;
CD: commercial diet.

Figure 4 Relative Δ6-desaturase (D6D) and Stearoyl-CoA desaturase (SCD) tissue-specific gene expression in non-transgenic common carp (*Cyprinus carpio*) fed on two different diets.

3.7 Comparasion of D6D and SCD mRNA level between transgenic and non-transgenic fish fed commercial diet

mRNA level of D6D and SCD gene varied ($P<0.05$) in muscle, liver, brain and gonad between transgenic and non-transgenic fish fed the commercial diet (Fig. 5). D6D and SCD mRNA level in muscle of transgenic fish were up-regulated ($P<0.05$) 12.7-fold and 17.9-fold, respectively, compared to that of non-transgenic fish. D6D mRNA level in the gonad of transgenic fish was up-regulated 6.9-fold ($P<0.05$) compared to that of non-transgenic fish. In contrast, D6D and SCD mRNA level in the brain of transgenic fish were dramatically down-regulated 50.2-fold and 16.7-fold ($P<0.05$), respectively, compared to non-transgenic fish. D6D and SCD mRNA level in liver of transgenic fish was down-regulated ($P<0.05$) 5.4-fold and 2.4-fold, respectively, compared to non-transgenic fish.



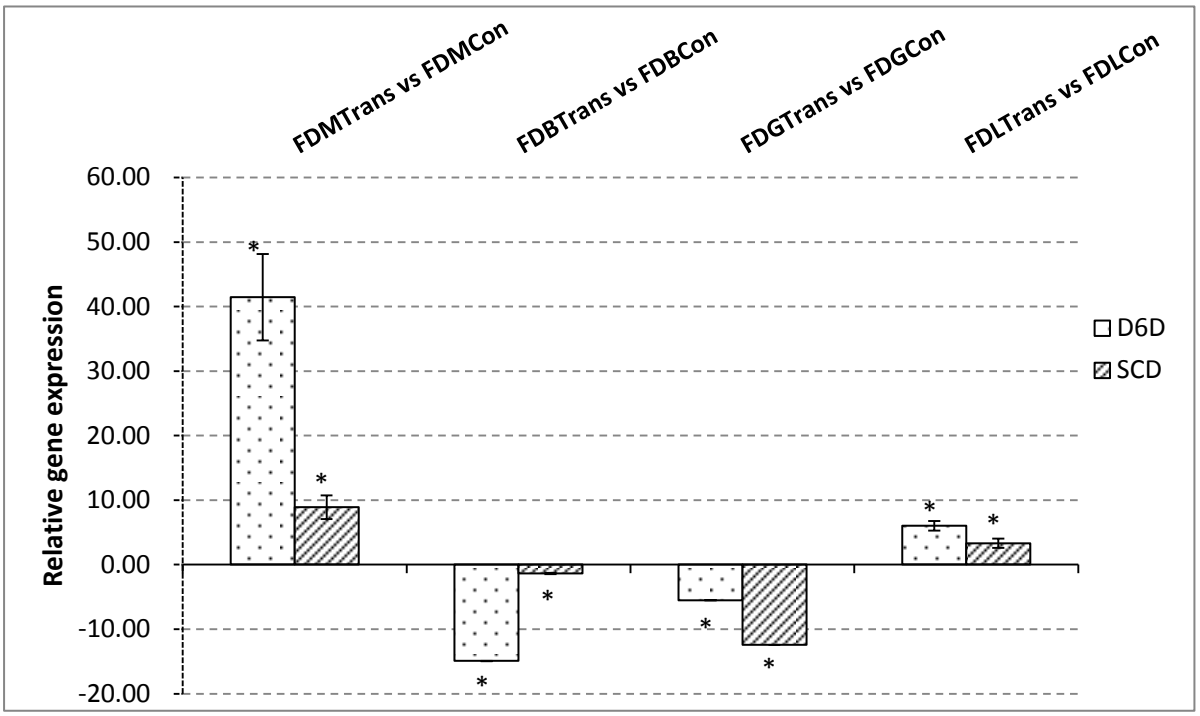
Relative gene expression was expressed as fold change of different tissues (liver, gonad, brain, muscle) in transgenic common carp fed commercial diet over that in non-transgenic samples as normalized to the expression of common carp (*C. carpio*) 18s ribosomal mRNA. Relative fold changes were expressed as mean ± SE. The * on the error bar indicated the relative expression between transgenic fish and non-transgenic fish fed commercial diet was significantly different from each other at $P < 0.05$ using Pairwise Fixed Reallocation Randomization Test (PFRR).

CDMTrans: muscle tissue of transgenic fish fed commercial diet; CDMCon: muscle tissue of non-transgenic fish fed commercial diet.
CDBTrans: brain tissue of transgenic fish fed commercial diet; CDBCon: brain tissue of non-transgenic fish fed commercial diet.
CDGTrans: gonad tissue of transgenic fish fed commercial diet; CDGCon: gonad tissue of non-transgenic fish fed commercial diet.
CDLTrans: liver tissue of transgenic fish fed commercial diet; CDLCon: liver tissue of non-transgenic fish fed commercial diet.

Figure 5 Relative gene Δ6-desaturase (D6D) and Stearoyl-CoA desaturase (SCD)'s expression in transgenic and non-transgenic common carp (*Cyprinus carpio*) fed commercial diet.

3.8 Comparison of D6D and SCD mRNA level between transgenic and non-transgenic fish fed fomulated diet

mRNA level of D6D and SCD gene varied ($P<0.05$) in muscle, liver, brain and gonad between transgenic and non-transgenic fish fed the formulated diet (Fig. 6). D6D and SCD mRNA level in muscle of transgenic fish were up-regulated ($P<0.05$) 41.5-fold and 8.9-fold, respectively, compared to that of non-transgenic fish. D6D and SCD were also up-regulated in liver of transgenic fish, 6.0-fold and 3.3-fold ($P<0.05$), respectively, compared to non-transgenic fish. D6D and SCD mRNA level in the gonad of transgenic fish were down-regulated 5.5-fold ($P<0.05$) and 12.4-fold ($P< 0.05$) respectively compared to that of non-transgenic fish. Additionally, D6D and SCD mRNA level in the brain of transgenic fish were down-regulated 14.9-fold and 1.4-fold ($P<0.05$), respectively, compared to non-transgenic fish.



Relative gene expression was expressed as fold change of different tissues (liver, gonad, brain, muscle) in transgenic common carp fed formulated diet over that in non-transgenic samples as normalized to the expression of common carp (*C. carpio*) 18s ribosomal mRNA. Relative fold changes were expressed as mean ± SE. The * on the error bar indicated the relative expression between transgenic fish and non-transgenic fish fed formulated diet was significantly different from each other at the level of $p < 0.05$ using Pairwise Fixed Reallocation Randomization Test (PFRR).

FDMTrans: muscle tissue of transgenic fish fed formulated diet; FDMCon: muscle tissue of non-transgenic fish fed formulated diet.
FDBTrans: brain tissue of transgenic fish fed formulated diet; CDBCon: brain tissue of non-transgenic fish fed formulated diet.
FDGTrans: gonad tissue of transgenic fish fed formulated diet; FDGCon: gonad tissue of non-transgenic fish fed formulated diet.
FDLTrans: liver tissue of transgenic fish fed formulated diet; FDLCon: liver tissue of non-transgenic fish fed formulated diet.

Figure 6 Relative gene Δ6-desaturase (D6D) and Stearoyl-CoA desaturase (SCD)'s expression in transgenic and non-transgenic common carp (*Cyprinus carpio*) fed formulated diet.

3.9 Fatty acid profile of transgenic and control common carp muscle fed two different diets

For the fish fed the formulated diet, there was no difference between the transgenic and control fish for any fatty acid variable measured (P=0.05, Table 6). The commercial diet produced higher (P=0.05) values than the formulated diet in the common carp for 16:0, 20:5n-3, 22:5n-3 fatty acids, HUFA, and total n-3 FA, while the formulated diet produced higher values (P=0.05) for 18:2n-6, 18:3n-3 FA, PUFA and total n-6 FA. In the case of fish fed the commercial diet, there was a trend for levels of specific FAs in the muscle with the transgenic common carp having a higher observed content of EPA, 1.07-fold (3.60 mg g^{-1} wet tissue versus 3.37 mg g^{-1} wet tissue), DPA was 1.12-fold (1.26 mg g^{-1} wet tissue versus 1.13 mg g^{-1} wet tissue), DHA was 1.07-fold (5.52 mg g^{-1} wet tissue versus 5.15 mg g^{-1} wet tissue) and that of total n-3 was 1.07-fold (12.65 mg g^{-1} wet tissue versus 11.80 mg g^{-1} wet tissue) than those in non-transgenic fish, although none were significantly different (P=0.05). However, this trend was not observed for fish fed the formulated diet. Total n-3 FA in all fish muscles was higher than n-6 FA, 2-fold (11.24 mg g^{-1} wet tissue versus 5.63 mg g^{-1} wet tissue) in control fish fed the formulated diet, 1.9-fold (10.87 mg g^{-1} wet tissue versus 5.73 mg g^{-1} wet tissue) in transgenic fish fed the formulated diet, 2.4-fold (11.80 mg g^{-1} wet tissue versus 4.93 mg g^{-1} wet tissue) in control fish fed the commercial diet, and 2.5-fold (12.65 mg g^{-1} wet tissue versus 5.05 mg g^{-1} wet tissue) in transgenic fish fed the commercial diet. FA in the muscle of common carp fed commercial diet had a higher content of EPA, 1.18-fold (3.37 mg g^{-1} wet tissue versus 2.86 mg g^{-1} wet tissue), DPA, 1.11-fold (1.13 mg g^{-1} wet

tissue versus 1.02 mg g^{-1} wet tissue) and total n-3, 1.05-fold (11.80 mg g^{-1} wet tissue versus 11.24 mg g^{-1} wet tissue) than those in fish fed formulated diet (Table 7).

Table 7 Total lipid (%) and fatty acid composition (mg g-1 diet and % of fatty acid methyl ester, FAME) of P1 Masou salmon Δ5-desaturase-like transgenic and control common carp, *Cyprinus carpio*, muscle fed on formulated diet (transgenic P1 for gene and control) and commercial diet (N=10 for formulated diet control; N=6 for formulated diet transgenic; N=10 for commercial diet control; N=7 for commercial diet transgenic)[1].

Selected Fatty Acid	Formulated Diet Control	Formulated Diet Transgenic	Commercial Diet Control	Commercial Diet Transgenic
Total lipid (%)	3.25[a]	3.26[a]	3.32[a]	2.90[a]
mg g^{-1} wet weight				
16:0	10.36[b]	10.28[b]	11.42[a]	11.58[a]
18:0	2.65[a]	2.70[a]	2.69[a]	2.73[a]
18:1n-9	11.97[a]	11.67[a]	11.92[a]	11.93[a]
18:2n-6	5.25[a]	5.34[a]	4.57[b]	4.63[b]
18:3n-3	0.59[ab]	0.60[a]	0.52[b]	0.52[b]
20:4n-6	0.06[b]	0.06[b]	0.06[b]	0.07[a]
20:5n-3	2.86[b]	2.80[b]	3.37[a]	3.60[a]
22:5n-3	1.02[c]	1.00[c]	1.13[b]	1.26[a]
22:6n-3	5.15[a]	4.82[a]	5.15[a]	5.52[a]
Saturates[2]	21.91[a]	22.39[a]	22.74[a]	23.88[a]
Monounsaturates[3]	19.57[a]	19.27[a]	20.93[a]	21.25[a]
PUFA[4]	8.28[a]	8.43[a]	7.67[a]	7.93[a]
HUFA[5]	9.65[b]	9.22[b]	10.33[ab]	11.07[a]
Total n-3[6]	11.24[b]	10.87[b]	11.80[ab]	12.65[a]
Total n-6[7]	5.63[a]	5.73[a]	4.93[b]	5.05[b]
FAME %				
16:0	17.41[c]	17.32[c]	18.54[a]	18.10[b]
18:0	4.46[a]	4.60[a]	4.36[a]	4.26[a]
18:1n-9	20.09[a]	19.44[a]	19.36[a]	18.65[a]
18:2n-6	8.81[a]	8.96[a]	7.42[b]	7.22[b]
18:3n-3	1.00[a]	1.01[a]	0.83[b]	0.80[b]
20:4n-6	0.10[b]	0.11[ab]	0.10[b]	0.12[a]
20:5n-3	4.82[b]	4.72[b]	5.46[a]	5.59[a]
22:5n-3	1.74[c]	1.69[c]	1.88[b]	1.97[a]
22:6n-3	8.68[a]	8.21[a]	8.35[a]	8.59[a]
Saturates[2]	36.98[a]	38.03[a]	36.85[a]	37.23[a]
Monounsaturates[3]	32.87[a]	32.14[a]	33.96[a]	33.20[a]
PUFA[4]	13.92[a]	14.19[a]	12.44[b]	12.34[b]
HUFA[5]	16.23[ab]	15.64[b]	16.75[a]	17.23[a]
Total n-3[6]	18.92[a]	18.44[a]	19.14[a]	19.70[a]
Total n-6[7]	9.46[a]	9.62[a]	8.01[b]	7.87[b]

[1] Values represent the average of triplicates samples per individual. Means within rows with the same letter are not significantly different (Duncan's Multiple Range Test, P=0.05)

3.10 Growth of transgenic and non-transgenic common carp

There was no difference (P>0.05) in body weight between transgenic and non-transgenic fish for either diet. Additionally, the tissue distribution of transgene did not affect (P<0.05) the body weight of the fish.

4. Discussion

Both dietary regulation and transgenic modulation were evaluated to biotechnologically enhance n-3 fatty acids (FA) production in a freshwater fish, common carp, *Cyprinus carpio*. The n-3 FA, primarily EPA and DHA, have gained worldwide attention since their beneficial functions on the membrane fluidity (Skrzypski et al., 2009), cell signaling (Skrzypski et al., 2009), organic development (Sargent et al., 2003), curing of cardiovascular disease (Calder and Yaqoob, 2009) as well as diseases resistance for arthritis, nephritis multiple sclerosis (Kremer et al., 1987, Bates et al., 1989) were identified. Marine fish has played an important role for the nutrition uptake of human and animals (Sargent and Tacon, 1999, Simopoulos, 1999) as the primary resource of EPA and DHA. However, with the decline in total capture of marine fish, marine fishing can no longer satisfy the need for EPA and DHA without aquaculture production and innovative biotechnological applications.

4.1 Generation of transgenic fish

In the present study, the transgene was distributed in all four tissues screened, liver, muscle, brain and gonad. In some cases, the transgene was found in 2 tissues. When exogenous DNA is introduced to a fish gamete or embryo, integration always occurs at the 2-cell stage or later, thus, all P1 transgenics are mosaics

(Stuart et al., 1990, Gross et al., 1992, Moav et al., 1993). The transgenic organism is always mosaic both among tissues and within cells in a tissue. Integration site can affect the level of expression of the transgene.

Approximately, 50% of the fish were transformed in at least one tissue. The rate of transformation varied among tissues. Fortunately, a high percentage of the individuals were transgenic in the gonad, 26.7%, which should allow transmission of the transgene to the next generation. Interestingly, the percentage of individuals transgenic for two tissues could be predicted by multiplying the transformation rate of each individual tissue. This has not been previously documented. If this independence principle holds true, the probability of detecting an individual transgenic in 3 tissues would be 0.5%, and indeed none out of 60 common carp were transgenic for 3 tissues. The results of Alimuddin et al (2006) using the same construct with zebrafish, but using microinjection were different than those of the current study. Approximately, 50% were transgenic in the P1 generation by examination of caudal fins. If they had examined more tissues, the percentage of mosaic P1 transgenics would have approached 100%. Exactly 25% of these fish transgenic in the caudal fin were also transgenic in the germline. If the independent principle for transformation rates in multiple tissues also applies to zebrafish, 25% of the fish that did not have the construct in the fin should have had the transgene in their germline, so the number of transgenic founders could have doubled with complete screening or mating. Identical results were obtained by Alimuddin et al (2005) for zebrafish microinjected with delta 6 desaturase gene except the

transformation rate in the fin was much lower, 14.7%, compared to 50% in the experiment above. However, the ratio, caudal fin transgenics/ transgenic in both caudal fin and germline was identical, 4.0, in both zebrafish studies. With this level of repeatability, an individual laboratory may be able to predict the value of screening or mating individuals negative for the primary screening tissue, which is usually fin. There must be a developmental reason for the consistent ratio of the transgene between tissues, and this should be explored in other species and for other transgenes.

4.2 Expression of D6D and SCD

Expression of D6D and SCD was not different ($P>0.05$) among liver, brain and gonad of non-transgenic common carp fed a commercial diet. However, expression of D6D and SCD was approximately 6-12-fold less in muscle compared to these other tissues.

Dietary treatment determines the synthetic abilities of C_{20} and C_{22} PUFAs in fish. The consumption of other marine fish is the source of C_{20} and C_{22} PUFAs for marine fish. Thus, certain marine fish have poor ability to synthesize C_{20} and C_{22} PUFAs. For example, turbot, lacks C_{18} to C_{20} elongase enzyme resulting in deficiency in the desaturation/elongation fatty acid pathway (Ghioni et al., 1999). In many marine fish such as cod, sea bass, etc., the functional activity and gene expression of D6D is barely detectable (Tocher et al., 2006b, Gonzalez-Rovira et al., 2009) , which was

not the case in the common carp as D6D expression was found in all 4 tissues evaluated.

The D6D transcription could be observed in a wide array of tissues, including the liver, brain, muscle and gonad. Results from the current study and others indicate that the D6D gene expression has an extensive range of tissue distribution (Vagner et al., 2009b). Currently four isoforms of SCD have been identified in the tissue-specific fashion of rodents and fish. SCD-1 is commonly found in adipose tissues and liver (Ntambi et al., 1988), SCD-2 is mostly expressed in brain (Kaestner et al., 1989), SCD-3 is specifically detected in skin (Zhang et al., 2001) and SCD-4 is abundant in heart (Miyazaki and Ntambi, 2003). However, Chang et al (2001) found that SCD was expressed only in the brain and liver in the grass carp. In the current study with common carp, a related cyprinid, the gene expression of SCD was observed not only in liver and brain, but also in muscle and gonad although the expression of SCD in muscle and gonad was relatively lower than in liver and brain in non-transgenic common carp fed commercial diet. The gene expression of SCD in these related species is different. Also, Polley et al (2003) observed that the transcripts of the SCD were only found in liver of common carp which is inconsistent with our result. Diets can partially explain different results.

PUFA and HUFA are high in fish oil (FO) and low in vegetable oil. Consumption of dietary PUFA and HUFA could reduce the D6D and SCD gene expression. Some studies have shown that high PUFA and HUFA can result in low expression of D6D and SCD in sea bass (Vagner et al., 2007a, Vagner et al., 2007b, Gonzalez-Rovira et

al., 2009, Vagner et al., 2009b). For example, higher D6D mRNA levels were observed in the sea bass larvae fed a low HUFA diet (0.5% or 0.7% EPA+DHA) than a high HUFA diet (1.7% or 3.7% EPA+DHA) (Vagner et al., 2007b). Similarly, González-Rovira et al (2009) reported that the D6D mRNA level in the liver of sea bass fed linseed and rapeseed oil was higher than fish fed FO. The regulation of desaturases by PUFA is related to two transcription factors, sterol regulatory element binding protein (SREBP) and peroxisome proliferator activated receptor-α (PPARα) (Nakamura and Nara, 2004a). PUFA suppresses the desaturases by binding to sterol regulatory element (SRE), which promotes the expression of many genes such as SCD, fatty acid desaturase, D6D and elongase. PPARα is responsible for the FA oxidation (Nakamura and Nara, 2004a).

In liver of non-transgenic common carp, the PUFA appears to have the same effect as in sea bass, and D6D and SCD expression decreased when the diet was changed from commercial diet to formulated diet. However, the data on the common carp indicates variable response to the increased PUFA in different tissues. The opposite effect occurred in muscle compared to liver. SCD transcripts in muscle increased after the diet were changed from commercial diet to formulated diet, and those for D6D were unchanged. In the case of gonad and brain, D6D and SCD transcript were not different between fish fed formulated diet and commercial diet.

There may be three explanations for the lower D6D and SCD gene expression in common carp fed formulated diet. The PUFA in the formulated diet was 2.7-fold higher than in the commercial diet, reaching a PUFA threshold level that blocks the

D6D enzymatic activity by binding to sterol regulatory element (SRE). Starvation may cause low expression of D6D and SCD (Oshino and Sato, 1972). Since the formulated diet only provided basic substrates without certain nutritional elements, this may be analogous to a starvation of key nutritional components resulting in both decreased D6D and SCD gene expression, as body weight gain of fish fed commercial diet was 1.54-fold higher (4 g/fish versus 2.6 g/fish) than fish fed formulated diet. The fatty acid composition in diet affected the growth of the fish. Several studies have revealed that high C18:3n-3 and C18:2n-6 in the diets creates more D6D production (Bell et al., 1993, Tocher et al., 2002, Izquierdo et al., 2008). However, excessive C18:3n-3 in the diet may decrease D6D transcription (Izquierdo et al., 2008). In the current study, 18:3n-3 was approximately 6 times greater in the formulated diet than the commercial diet, which could lead to a stronger inhibition of D6D expression in the formulated diet.

SCD mRNA level in the muscle of non-transgenic fish fed formulated diet was higher compared to that of commercial diet which is opposite of the expression in liver. Perhaps, threshold levels of PUFA and HUFA differ among tissues, and promoter response varies in different tissue and transcriptomic environments. Tissue-specific D6D and SCD expression indicates different regulatory mechanisms, and may reflect different physiological requirements for these fatty acids in different tissues and organs. The results in our current study and those documented from other studies suggest that the nutritional regulation of D6D and SCD could vary according to the species or the developmental stage considered (Tocher et al., 2006b).

Several studies document the variable effect of temperature on desaturation activity in different tissues. For example, the highest desaturation activity was observed in enterocytes at 7 °C and in hepatocytes at 11 °C for rainbow trout (Tocher et al., 2004, Skalli et al., 2006). Thus, the relative tissue-specific gene expression among various species is likely influenced by temperature.

4.3 Effect of the transgene on D6D and SCD expression

mRNA level of D6D and SCD gene varied in muscle, liver, brain and gonad between transgenic and non-transgenic fish fed the commercial diet. D6D and SCD mRNA level in muscle of transgenic fish were up-regulated 12.7-fold and 17.9-fold, respectively, compared to that of non-transgenic fish. This up-regulation in muscle was even more dramatic for D6D, 41.5 X, but not SCD, 8.9X when the fish were fed the formulated diet. D6D and SCD mRNA level in the brain of transgenic fish were down-regulated 14.9-fold and 1.4-fold, respectively, when fed the formulated diet, but were much more dramatically down-regulated 50.2-fold and 16.7-fold, respectively, when fed the commercial diet.

There were additional genotype X tissue X diet interactions. D6D and SCD mRNA level in liver of transgenic fish was down-regulated 5.4-fold and 2.4-fold, respectively, compared to non-transgenic fish when fed the commercial diet. However, D6D and SCD were up-regulated in liver of transgenic fish, 6.0-fold and 3.3-fold, respectively, compared to non-transgenic fish when fed the formulated diet. Also, D6D mRNA level in the gonad of transgenic fish was up-regulated 6.9-fold

compared to non-transgenic common carp when fed the commercial diet, while D6D and SCD mRNA level in the gonad of transgenic fish were down-regulated 5.5-fold and 12.4-fold when fed the formulated diet. The desaturase/elongase activities involved in the early desaturation/elongation pathway have a remarkable effect for the later production of EPA and DHA (Ghioni et al., 1999). The tissue environment affects the expression of the transgene, and the differential gene expression of other genes could be affecting the transgene expression via epistasis.

In the case of D6D transgenic zebrafish, the greatest expression of the transgene was highest in the gill > liver>intestine and brain >eye >muscle and fin (Alimuddin et al., 2005). The reaction of different species to the same or similar transgenes may vary. The results of the current study are not in total agreement with the zebrafish data as the D5D transgenic common carp had the highest expression of D6D in the gonad, liver and muscle expression was similar and expression in the brain was the lowest. This illustrates that model organisms do not necessarily give results that would be 100% congruent with primary species of interest.

4.4 Growth of common carp on diets with varying fatty acid and effect of the transgene

The body weight gain of the non-transgenic common carp on the commercial diet was approximately 6.2 g and on the formulated diet was 5.0 g. Individual measurements prior to this growth phase were not taken precluding statistical analysis on weight gain. However, assuming normal individual variation with a coefficient of variation of 30, fish on the commercial diet were growing faster. This

difference in weight gain could be due to the fatty acid composition or it may be a result of other nutritional differences between the two diets, which were dramatic in regards to protein, fat and vitamin content. However, Ren et al. (2012) found no effect of n-6 or n-3 fatty acids on common carp growth rate, thus other nutritional differences in the diets may be the more likely cause of any growth differences.

The transgenic and non-transgenic common carp did not differ in body weight for either diet. Thus, the Masou salmon D5D transgene had no pleiotropic effect on growth in the P1 generation of transgenic common carp. This is important for any future commercial application of desaturase transgenic fish as adverse effects on other important production traits would decrease economic value of the fish. The tissue that was found transgenic had no discernible effect on body weight, which would likely indicate no adverse effects would be predicted for growth in F1 desaturase transgenic common carp that would have the transgene in every cell and tissue.

In contrast to our results, Alimuddin et al. (2006) found that F2 transgenic zebrafish males containing the Masou salmon D5D construct grew 6-18% faster than controls, a positive pleiotropic effect. Perhaps, the same positive impact on growth will be found in the F1 generation of transgenic common carp.

Again, the interaction between the transgene and the diet is important, as diet and fatty acid content affected the magnitude of the growth difference in the Masou salmon D5D transgenic zebrafish males. The results from the current study support this observation. The observed body weight of transgenic common carp was 25.8%

larger for the commercial diet compared to the formulated diet, which approached significance. In both the zebrafish and common carp example, the desaturase 5 transgenic fish grow better on the diets with higher n-3 fatty acids, although in both cases other nutrient components cannot be ruled out as effectors.

4.5 Effect of diet and transgene on n-3 fatty acid level

The muscle FA composition of both transgenic and non-transgenic fish fed two different diets was also investigated. The EPA, DPA, DHA and n-3 FA in muscle of transgenic common carp fed the commercial diet were higher than that of non-transgenic fish. The strategy of utilizing genetically engineered fish has the potential to increase valuable FA in farmed freshwater fish that are normally low in n-3 FA. However, the content of EPA, DPA, DHA and total n-3 FA of transgenic fish fed the formulated diet tended to decrease although not significantly. Thus, the genetic engineering must be coupled with the appropriate nutritional input to achieve the goal of increasing n-3 FA in the edible muscle.

Common carp fed the commercial diet had a higher content of total n-3, EPA, DPA and DHA. In the current study, the DHA, EPA, DPA and total n-3 are all much higher in the commercial diet than the formulated diet. Both control and transgenic common carp had higher levels of EPA, DPA, DHA and n-3 in their muscle when fed the commercial diet. Likely, they are absorbing these FAs directly from the diet. Bell et al. (Bell et al., 2002) reported that when the ratio of constitute VO/FO more than 1/2 of the dietary lipid, a reduction of EPA, DHA and n-3/n-6 FA in muscle

resulted. The HUFA such as EPA and DHA in VO is extraordinarily less than that in FO.

Tocher et al. (1997) showed that C18:3n-3 desaturation products increased in hepatocytes of Atlantic salmon fed diets contained high levels of n-6 FA. This is similar to results in the current study as the C18:2n-6 levels are 4.6× and 6.6× higher than C18:3n-3 in formulated diet and commercial diet, respectively, however the opposite is found in the common carp muscle as n-3 FA was 1.9 to 2.5-fold higher than n-6 FA for fish fed the formulated and commercial diet. This indicates that the D6D has higher affinity to n-3 FA than n-6 FA.

F2 transgenic zebrafish containing D5D expressed D5D in their caudal fin, but variably from one individual to another (Alimuddin et al., 2005). In many cases, the level of expression varied more than 10-fold from one individual to another. The transgenic individuals had 13-21% increase in n-3 fatty acids in their whole body compared to controls. Although not statistically significant, the P1 D5D transgenic common carp had an observed mean 7.2% higher for n-3 fatty acids in their muscle compared to the control. These P1 are mosaic, and likely only have the transgene in 10-50% of their cells in the muscle. If the n-3 fatty acids in the P1 transgenics are being diluted proportionately, one might predict that the increase in n-3 fatty acid in the F1 could be 14-70% compared to the control. These F2 desaturase transgenic zebrafish had approximately 15 mg/g of n-3 fatty acids in their whole body, while the P1 transgenic common carp contained approximately 9 mg/g in their muscle.

Alimuddin et al. (2005) also produced D6D transgenic zebrafish. Again, large variation existed from one transgenic line to another resulting in a 40-110% increase in n-3 fatty acids.

Rahman et al (1998) reported that F1 Nile tilapia transgenic for a construct consisting of a sockeye salmon metallothionein promoter spliced to a sockeye salmon growth hormone gene exhibited no growth enhancement, although salmon transgenic for this construct show greatly enhanced growth. It is the usual case to have variation in response to transgene insertion among species and families (Dunham and Liu, 2006). This variation may be related to position effect, copy numbers, genetic background. Theoretically, selection of the best performing transgenic lines should maximize genetic enhancement for the trait of interest.

Although the percent improvement (40-100%) in n-3 fatty acids using D6D transgenesis in zebrafish (Alimuddin et al., 2005) was more impressive than D5D transgenesis (13-21%), the level of n-3 fatty acids in the whole body of D6D transgenics,7 mg/g, was less than that for D5D transgenics, 15 mg/g. The large influence of diet on n-3 fatty acid deposition in the flesh must be considered when evaluating the effectiveness and application of desaturase transgenesis.

Genetic improvement and dietary regulation can generate fish that can synthesize increased amounts of EPA/DHA. Additionally, construct design with more effective promoters should be explored to further improve upon the results of the current study. F1 desaturase transgenic common carp need to be evaluated to determine D5D desaturase transgene expression and its effect upon fatty acid composition

when every cell in the organism has the capacity to produce more D6D and SCD. The potential of the gene transfer of desaturase cannot be realized without using the proper diet with the needed amount of substrates.

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