Targeted Gene Insertion and the Effect of Random Insertion of the Masou Salmon Delta5-Desaturase Transgene on n-3 Fatty Acid Production and Culture Traits of Channel Catfish (*Ictalurus punctatus*)

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
December 15, 2018

Keywords: fatty acid desaturase, omega-3 fatty acid,
Culture traits, channel catfish, transgenic, salmon

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Abstract

Channel catfish (Ictalurus punctatus) is one of the most abundant catfish species in North America. The objectives of this study were to determine the effectiveness of the Masou salmon delta5-desaturase transgene driven by a beta-actin promoter for improving n-3 fatty acid production in F1 transgenic channel catfish, Ictalurus punctatus together with proximate analysis, as well as to determine pleiotropic effects of disease resistance. Additionally, a novel approach of using multiple transgenes to alter multiple steps in a pathway, which has not been previously reported in aquaculture species was utilized. As a result, Apo14-Masousalmon_Elovl2 gene was successfully inserted on chromosome 1 in one channel catfish utilizing microinjection and CRISPR/Cas9 mediated knockin. Transgenic fish showed a 33% increase in n-3 fatty acid level, and a 15% decrease in n-6 fatty acids level. Masou salmon delta5-desaturase transgene driven by a beta-actin promoter affected n-3 fatty acid production in a positive direction. Insertion of the masou salmon delta5-desaturase transgene into channel catfish had major effects on metabolism, physiology, body composition, and growth of channel catfish. A major goal of increasing the percentage of n-3 fatty acids was achieved, but the objective of increasing total n-3 fatty acids was not. Body composition was altered, pleiotropic effects on performance traits were observed and the transgene appeared to increase uniformity of these traits.

Acknowledgements

I want to thank my major professor, Dr. Rex Dunham, for helping me and advising me during my time at Auburn University. I would also like to thank my committee members Dr. Butts and Dr. Chen for their feedback, as well as Dr. Davis and Dr. Salze for allowing me to use their nutrition laboratory. I also greatly appreciate the patient assistance of Dr. Bangs and thanks my lab mates, friends, and family for their assistance and encouragement in completing this project.

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List of Abbreviations

AA: Arachidonic Acid

ALA: α-linolenic Acid

CV: Coefficient of Variation

CVD: Cardiovascular Disease

DGA: Dihomo-γ- Linoleic Acid

DHA: Docosahexaenoic Acid

DPA: Docosapentaenoic Acid

EPA: Eicosapentaenoic Acid

ETA: Eicosatetraenoic Acid

ETE: Eicosatrienoic Acid

GH: Growth Hormone

LA: Linoleic Acid

PUFA: Polyunsaturated Fatty Acid

SD: Standard Deviation

FAME: Fatty Acid Methyl Ester

Introduction

Catfish is a nutrient-rich aquatic product, rich in lecithin, protein, essential amino acids, calcium, phosphorus, zinc, magnesium, iron, iodine, and other nutrient (Gary, n.d.). The main aquaculture species in the United States is catfish. Channel catfish (*Ictalurus punctatus*) is one of the most abundant catfish species in North America angled by approximately 8 million fishermen every year, which makes channel catfish one of the most fished species in the United States (Keenan, 2011; Carlander, 1969).

Since the early 1930's omega-3 fatty acids have been reported as essential for normal growth and human health (Holman, 1998). Omega-3 fatty acids have proved to be very effective for treatment of major depression disorders and other psychiatric disorders. Recently, omega-3 fatty acids have been used for the treatment of several mental disorders (Bozzatello, 2016). In neural phospholipids, highly unsaturated fatty acids (HUFAs) are concentrated strongly, and become important parts of the neuronal cell membrane. Some mechanisms of brain cell signaling such as the dopaminergic and serotonergic pathways can be modulated by HUFAs (Bozzatello, 2016). The mortality rate of sepsis and sepsis-induced ARDS can be reduced by supplementation of omega-3 fatty acids (Chen, 2018).

At one time, Humans consumed a 1:1 to 2:1 ratio of omega-6 to omega-3 fatty acids, but since the industrial revolution, the ratio has shifted towards 15:1 to 16.7:1 (Simopoulos, 2002). Experimental studies have suggested that omega-6 and omega-3 fatty acids have opposite effects on body fat gain through mechanisms of adipogenesis, browning of adipose tissue, lipid homeostasis, brain-gut-adipose tissue axis, and most importantly systemic inflammation (Simopoulos, 2002). The risk of obesity increases as the level of omega-6 fatty acids and the ratio increase in red blood cell membrane phospholipids, whereas high omega-3 red blood cell

membrane phospholipids decrease the risk. Not only the quantity of omega-6 and omega-3 fatty acid intake, but the omega-6/omega-3 ratio also increase the development of obesity through both arachidonic acid (AA) eicosanoid metabolites and hyperactivity of the cannabinoid system. However, by increasing intake of EPA and DHA, this situation can be reversed. Balancing omega-6/omega-3 ratio plays an important role in maintaining health,as well as preventing and controlling obesity (Simopoulos, 2016).

Polyunsaturated fatty acids refer to long fatty acid chains that have more than 16 carbon atoms and have at least two or more double bonds (Brett & Navarra 1997). The first double bond is between the third and fourth carbon atoms from the tail end in omega-3 fatty acids. α-linolenic acid (ALA) found in plant oils, and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both commonly found in marine oils are 3 types of omega−3 fatty acids involved in human physiology. The shorter-chain omega−3 fatty acid ALA can be obtained through diet and form long-chain omega−3 fatty acids, EPA, then from EPA, the most crucial, DHA.

Oils from marine fish are traditional sources of n-3 fatty acids, but are expensive. To produce these essential fatty acids and reducing their cost, freshwater fish with lower n-3 fatty acid levels are an alternative choice (Ugoala, Ndukwe, & Audu, 2008). Catfish only contain 151mg, while salmon and tuna average 1825mg and 733mg, respectively, of EPA and DHA per 85g serving. When accounting for all n-3 fatty acids, there are 2.15g per 100g of salmon (*Oncorhynchus kisutch*) in contrast to 0.6g per 100g tissue in carp (*Oncorhynchus mykiss*) and 0.28g per 100g tissue in catfish (*Ictalurus punctatus*) (Nettleton, 2000; Gebauer, Psota, Harris, & Kris-etherton, 2006; Cheng et al., 2014).

AA yields highly inflammatory products through omega-6 fatty acid metabolism while EPA metabolism tends to be much less potent to form omega-3 metabolites (Das, 2006). Both

cyclooxygenase and 5-lipoxygenase are required for these 2 competing metabolic processes. EPA results in a more n-3 dominated pathway, but increasing levels of LA in blood plasma can shift enzyme metabolism from ALA and n-3 metabolism towards the n-6 pathway (Ruxton et al., 2004).

Production of n-3 fatty acids relies on delta 5 and delta 6 desaturases as well as elongase function. Delta desaturase is an enzyme that can replace two hydrogen atoms at a certain point with a double bond. Delta 5 and delta 6 are membrane bound desaturases, delta 5 creates a double bond at the fifth position from the carboxyl end while delta 6 creates one at the 6th position. Elongase is an enzyme that can catalyze delta 5 and delta 6 desaturation by adding two carbon atoms into the fatty acid chain (Guillou et al., 2010). In some organisms, linoleic acid (18:2 n-6) can be produced from oleic acid (18:1 n-9) through delta 12-desaturation, then form ALA through delta-15 desaturation (Lee et al., 2016). However, channel catfish lack necessary desaturases, and they only obtain delta 5 and 6 desaturase precursors, 18:2 n-6 linoleic acid or 18:3 n-3 α-linolenic acid, from their diets (Hastings et al., 2001).

In polyunsaturated fatty acid formation, delta 6 desaturase uses linoleic acid (18:2 n-6) or α – linolenic acid (18:3 n-3) to produce either γ – linoleic acid (18:3 n-6) or stearidonic acid (18:4 n-3) first. Then two carbon atoms were added onto the chains by elongase and from dihomo-γ-linoleic acid (DGA) (20:3 n-6) or eicosatetraenoic acid (ETA) (20:4 n-3). Finally, these products can form arachidonic acid (AA) 20:4 n-6 or eicosapentaenoic acid (EPA) 20:5 n-3 via delta-5-desaturase.

Eicosanoids are also produced after the formation of dihomo-γ-linoleic acid (DGA) (20:3 n-6) or eicosatetraenoic acid (ETA) (20:4 n-3). Production of AA leads to formation of 2-series prostaglandins, 2-series thromboxanes, and 4-series leukotrienes while production of EPA results

in 3-series prostaglandins, 3-series thromboxanes, and 5-series leukotrienes (Wall et al., 2010), productions of EPA are less inflammatory compared to production of AA (Simopoulos, 2002).

Next, multiple elongase steps can occur (Figure 1). If n-6 fatty acid is metabolized first, AA will produce docosatetraenoic acid 22:4 n-6, and then tetracosaetetaenoic acid 24:4 n-6 is formed. If n-3 fatty acids are metabolized first, elongation forms docosapentaenoic acid (DPA) 22:5 n-3, followed by tetracosapentaenoic acid 24:5 n-3. Delta-6-desaturase will metabolize these secondary elongation productions to form tetracosapentaenoic acid 24:5 n-6 or tetracosahexaenoic acid 24:6 n-3. Then β-oxidation will be achieved by peroxisomes, producing docosapentaenoic acid 22:5 n-6 from omega-6 fatty acids, or docosahexaenoic acid 22:6 n-3 DHA from omega-3 acids (Nakamura and Nara, 2004; Wall et al., 2010).

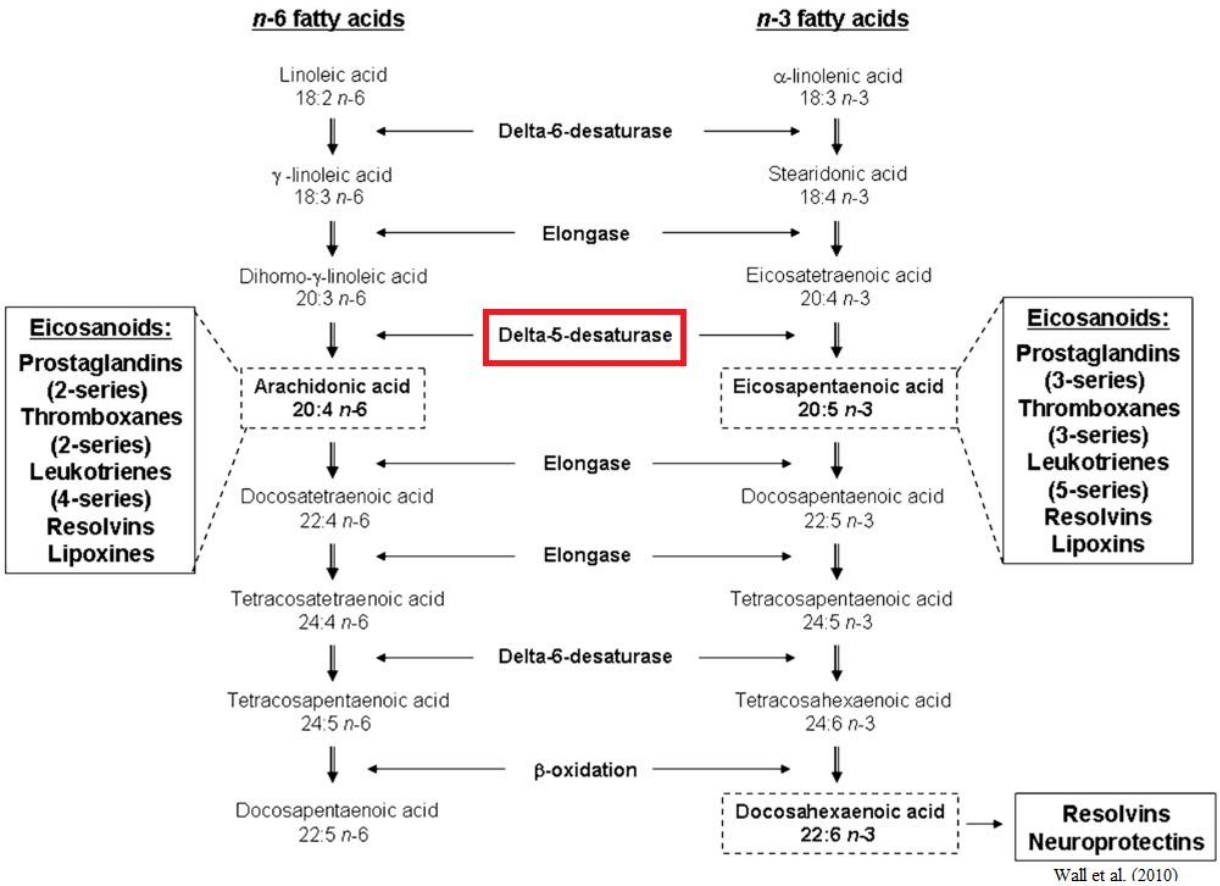

Figure 1: Vertebrate n-6 and n-3 fatty acid desaturation pathway responsible for the production of necessary polyunsaturated fatty acids (PUFAs) (Wall et al., 2010).

Genetic engineering is a potential method to produce fish capable of enhanced n-3 fatty acid production as well as other traits. Performance traits have been altered in fish via transgenesis. Growth hormone gene has been transferred to several species, including loach (*Misgurnus anguillicaudtis)*, common carp (*Cyprinus carpio*), crucian carp (*Carassius carassius*), Atlantic salmon (*Salmo salar*), channel catfish, Nile tilapia (*Oreochromis niloticus*), medaka (*Oryzias latipes*) and northern pike (*Esox lucius*) resulting in 10 to 3,000% faster growth rates compared to non-transgenic fish in aquaculture conditions (Dunham, 2004).

Third generation growth hormone (GH) transgenic common carp (TG) had 1.49 times higher mean body weight compared with the non-transgenic common carp (NT) (Kurdianto, 2016). Transgenic fish showed higher total feed consumption, survival, body protein content, protein and lipid retention, hepatosomatic index, and lower feed conversion ratio. However, body lipid content and blood glucose level of TG fish were lower than NT fish, and total ammonium nitrogen level in rearing media of TG fish was 51.78% lower (Kurdianto, 2016). Significantly increased growth rate was found in Atlantic salmon, when utilizing a chinook salmon growth hormone gene and an ocean pout promoter sequence. Growth rate of transgenic individuals was between 6 and 13 times greater than controls in 8-month-old smolt (Du et al., 1992; Fletcher et al., 1992). Three years later, transgenic individuals were reported to have about 10 times the size of their controls (Devlin et al., 1995; Cook et al., 2000). These results were all produced by random gene insertion. The direct and pleiotropic effects, positive and negative, of random gene insertion have been hypothesized, but the actual effects are unknown.

CRISPR technology now allows targeted gene insertion (Chu et al., 2016; Xu et al., 2014; Auer et al., 2014). Co-injection of Cas9 mRNA and target gene sgRNA into one-cell stage embryos has been demonstrated as an efficient and reliable approach to modify the genome of

different species. In 2013, the CRISPR/ Cas9 system was first successfully applied in mammalian genome editing (Cong et al., 2013). In 2014, scientists used CRISPR/Cas9 to generate the first gene knockout cynomolgus monkey (*Macaca fascicularis*) via targeting one-cell embryos (Niu et al., 2014). In the same year, scientists first coinjected Cas9 mRNA and sgRNAs into cynomolgus monkeys embryos and achieved precise gene targeting for genome editing in one-cell-stage (Chen et al., 2015; Ming et al., 2016). Jinhuan et al. (2015) fist obtained mice (*Mus*) with germline transmission of DNA fragment inversions by coinjecting embryos with Cas9 mRNA and a pair of sgRNAs. Now, CRISPR has been used to generate genetically modified mouse models and successfully generated large-scale genome-modified mice (Dow, 2015; Flynn et al., 2015; Mou et al., 2015; Fujii et al., 2013).

Moreover, CRISPR/Cas9 has been successfully applied in bacteria (Bikard et al., 2013; Selle et al., 2015), yeast (DiCarlo et al., 2015; Lee et al., 2015; Mans et al., 2015; Tsai et al., 2015), roundworm (*Caenorhabditis elegans*) (Friedland, 2013; Chen et al., 2014, 2015), fruit flies (*Drosophila*) (Bassett et al., 2013; Gratz et al., 2013), rice (*Oryza sativa*) (Xu et al., 2014, 2015; Zhang et al., 2014), zebrafish (*Danio rerio*) (Varshney et al., 2015), mouse (Shen et al., 2013), axolotl (*Ambystoma mexicanum*) (Flowers, 2014), Western clawed frog (*Xenopus tropicalis*) (Blitz, 2013; Nakayama, 2013), rat (*Rattus*) (Hu, 2013; Nakamura et al., 2014) and pig (*Sus*) (Whitworth, 2014) for genome editing in different species. CRISPR/Cas9 has also been used in arthropods (Sun et al., 2017), such as yellow fever mosquito (*Aedes aegypti*) (Dong et al., 2015; Hall et al., 2015), tobacco cutworm (*Spodoptera litura*) (Bi et al., 2016; Zhu et al., 2016), diamondback moth (*Plutella xylostella*) (Huang et al., 2016) and cotton bollworm (*Helicoverpa armigera*) (Wang J. et al., 2016; Khan et al., 2017; Chang et al., 2017) for genome editing.

One purpose of targeted gene insertion is to reduce the incidence of negative pleiotropic effects. Either positive or negative pleiotropic effects may be associated with transgene insertion. Increased feed conversion efficiency has been demonstrated in common carp, channel catfish, Nile tilapia, and loach (Chatakondi, 1995; Rahman et al., 2001; Dunham & Liu, 2002; Nam et al., 2004). rtGH transgenic common carp showed a longer average survival time at 0.4ppm dissolved oxygen than non-transgenic full siblings (Chatakondi, 1995; Dunham & Liu, 2002). Compared to controls, GH transgenic salmon showed higher oxygen demand in the same situation (Lee et al., 2003).

Transgenes can also affect reproduction and viability of progeny. GH transgenic male medaka fish showed a significant mating advantage over controls, and were involved in 76% of all mating events in tanks with even numbers of transgenic and control males in competition (Howard, Dewoody, & Muir, 2004). On the contrary, transgenic coho salmon demonstrated a decreased mating rate (Bessy et al., 2004). Offspring of the fastest growing transgenic salmon (30 times normal growth) are subjected to elevated levels of endocrine stimulation and have near zero survival (Devlin et al., 1995).

Alteration in disease resistance also can be a pleiotropic effect of transgenesis. Sometimes, transgenic fish show increased disease resistance. Zebrafish with delta 5 desaturase transgene have higher resistance to bacterial infection as they have greater immunomodulatory activity (Wang, Tan, Jiao, You, & Zhang, 2014). However, not all transgenic fish have higher disease resistance. Transgenic salmon showed higher sensitivity to *Vibrio* than controls (Jhingan et al., 2003).

Several negative pleiotropic effects have been observed when transgenes were integrated into fish genomes. Rapid growth accelerates telomere attrition in a transgenic fish (Pauliny,

2015), resulting in 1.4-fold EPA and 2.1-fold DHA in transgenic fish compared to controls. Morphological abnormalities have also been found in transgenic fish. Fast growing transgenic salmon as well as transgenic GH rainbow trout have increased cartilage and bone production, and demonstrated morphological abnormalities in the head, fin, jaw, and operculum (Devlin et al., 1995, Devlin et al., 2001). Gill filaments of transgenic Pacific salmon have the same length as controls, but decreased lamellar spacing (Stevens & Devlin, 2000). However, transgenic Atlantic salmon had similar lamellar spacing, but longer gill filaments than controls (Stevens & Sutterlin, 1999).

GH transgenic channel catfish showed increases in number of mitochondria, glycogen globules, muscle fibers, and a decrease in fat globules in cells which makes flavor and the texture of the transgenic catfish flesh altered compared to controls (Dunham & Liu, 2002). Growth hormone transgenic Amago salmon (*Oncorhynchus masou ishikawae*) showed both accelerated growth and altered fatty acid composition and content in liver tissues. They showed a decrease in saturated fatty acids and monounsaturated fatty acids, but an increase in polyunsaturated fatty acids not including docosahexaenoic acid (22:6n−3) and eicosapentaenoic acid (20:5n−3). Moreover, transgenic fish have increased levels of 3-hydroxybutyric acid and a decreased in serum glucose, triacylglycerol (Sugiyama, 2012).

In the case of desaturase transgenesis, delta-5 desaturase transgene P-1 common carp had 7.3% higher n-3 fatty acid production than controls (Cheng et al., 2014). Delta-5 desaturase transgenic F1 and F2 zebrafish showed 21% and 24% increased levels of EPA and DHA (Alimuddin et al., 2007), and demonstrated increased disease resistance and immunomodulatory activity when challenged with *Vibrio vulnificus*. Futhermore, transgenic zebrafish showed a 350% increase in survival and a decrease in bacterial counts during *Vibrio vulnificus* infection

(Cheng et al., 2015). Polyunsaturated fatty acids production, antibacterial and anti-inflammatory activity are enhanced in desaturase transgenic zebrafish muscle (Chih-Lun Cheng, 2015).

The first objective of this project was to evaluate the effectiveness of delta-5 desaturase transgene from Masou salmon, *Oncorhynchus masou*, for increasing omega-3 fatty acid production in F1 transgenic channel catfish, and determined pleiotropic effects of the transgene on body weight, body composition, low oxygen tolerance and disease resistance. Second objective was to initiate evaluation of utilizing CRISPR/Cas9 for targeted multiple transgene insertion to alter multiple steps in a methabolic pathway, the fatty acid pathway which has not been previously reported in aquaculture species was utilized.

Materials and Methods

Transgene Construct

FRm2bl-Δ5-desaturase plasmid (Figure 2) was built from a backbone construct (NCBI accession #: AF170915.1). The green-fluorescent protein sequence was removed and replaced with a 1.4kb *Oncorhynchus masou* delta5-desaturase-like gene (D5D, Accession ID: EU098126.1). The D5D transgene was flanked by a beta-actin promoter from *Cyprinus carpio* (Accession ID: M24113.1) that was used to drive D5D and an antifreeze polyA terminator from *Zoarces americanus* (Accession ID: S65567.1) to terminate transcription. This synthetic construct, totaling 8.3kb, was built by GenScript (USA Inc., Piscataway, NJ 08854, USA) (Cheng et al., 2014).

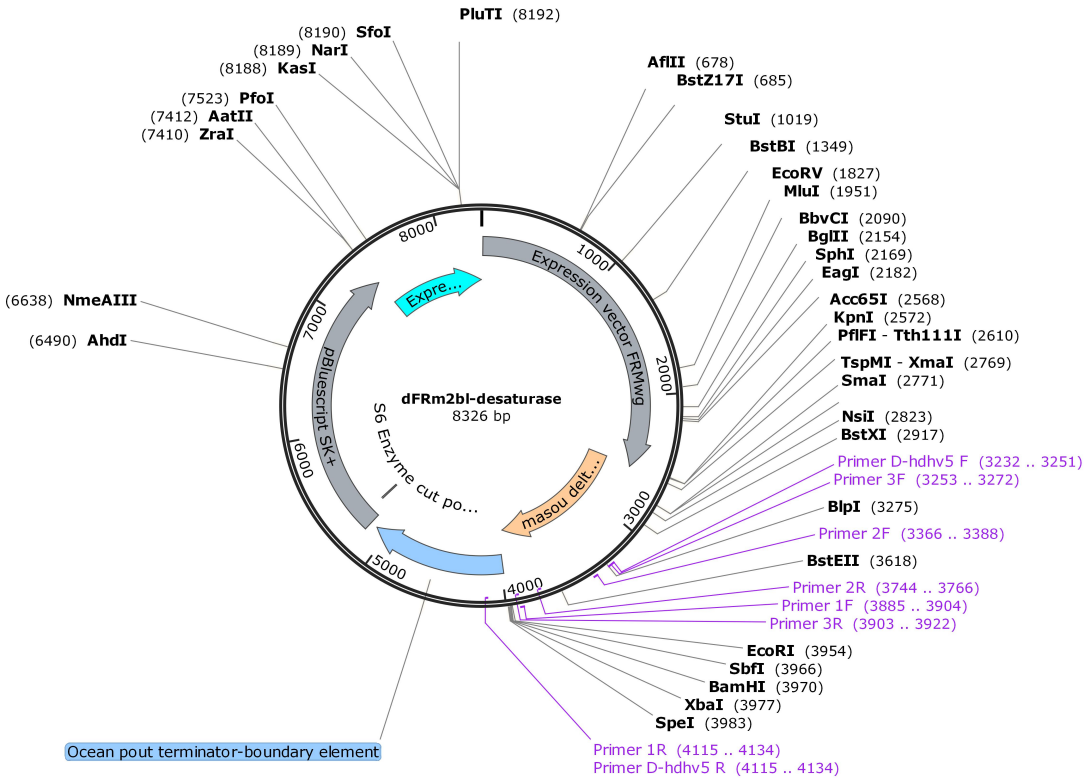

Figure 2: Restriction site map of the delta-5 desaturase transgene utilizing the *Oncorhynchus masou* delta-5 desaturase construct containing the beta actin promoter from *Cyprinus carpio.*

Donor β-action-elovl2 desaturase transgene plasmid (Figure 3). A new desaturase construct was designed by replacing the CherrySalmon_Δ5-desaturase gene with the Apo14-Masou salmon_Elovl2 gene based on the FRm2bl-Δ5-desaturase plasmid with carp β-action promoter, then the homologous arms of the channel catfish genomic DNA was added to the left of carp_β-action_promoter and the right of the ocean pout_antifreeze_polyA_terminator. The above plasmid was linearized by KpnI and EcoRI enzyme digestion and then the Cherry Salmon Δ5-desaturase gene was excised. The In-Fusion clone method was used to insert the Apo14-Masou salmon_Elovl2 gene into the plasmid. The homologous arm was synthesized from the company and In-Fusion clone method was used to insert the Carp_β-action_promoter/Apo14-Masousalmon_Elovl2/OceanPout_antifreeze_polyA_terminator sequences into a pUC57 plasmid backbone, then the donor plasmid was complete. This construct totaled 7.7kb.

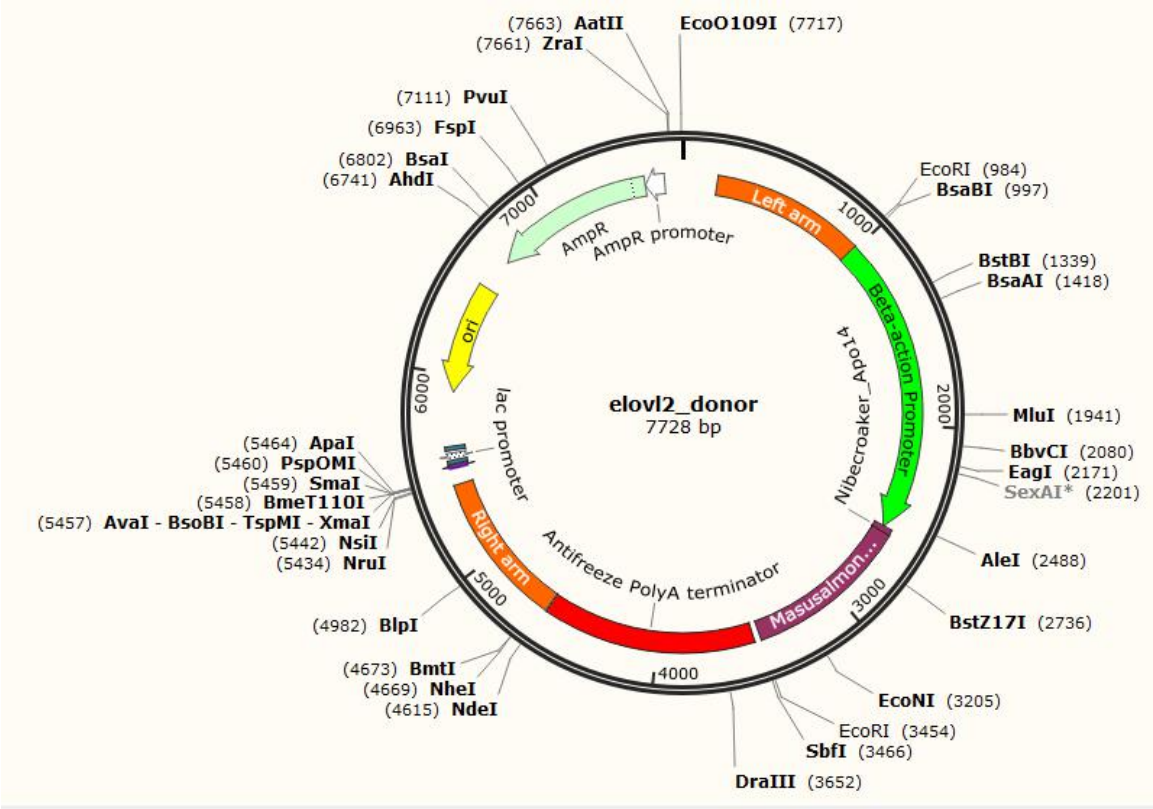

Figure 3: Restriction site map of the elovl2 desaturase transgene utilizing the Apo14-Masousalmon_Elovl2 plasmid containing the beta actin promoter from *Cyprinus carpio*.

Donor β-actin-Δ4 desaturase transgene plasmid. The donor delta4 desaturase transgene plasmid was built similarly as the donor elovl2 desaturase transgene plasmid by replacing the cherry salmon_Δ5-desaturase gene with the Apo14-rabbitfish_D4_desaturase gene based on the FRm2bl-Δ5-desaturase plasmid with carp β-action promoter. This construct totaled 8.2kb.

Donor β-actin-Δ5_6 desaturase transgene plasmid. The donor delta5/6 desaturase transgene plasmid was built similarly as the donor elovl2 desaturase transgene plasmid by replacing the cherry salmon_Δ5-desaturase gene with the Apo14-rabbitfish_D5/6_desaturase gene based on the FRm2bl-Δ5-desaturase plasmid with carp β-actin promoter. This construct totaled 8.2kb.

gRNA and Cas9 protein

The primers above were reconstituted by using DNase / RNase Free water to (100 mM) as stock solution and then the working solution (10 mM). The double-stranded DNA template for sgRNA synthesis were synthesized by adding 10.75µl dH2O, 2.5µl dntps, 2.5µl 10X buffer, 1.25µl MgCL2, 4µl common primer, 4µl sgRNA templating primer and 0.1µl Taq polymerase per run. The oligos were annealed and then mixed with the Taq polymerase for reaction in one step using the following PCR program: initial denaturation for 3 min at 94 °C; followed by 5 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 20 s, then followed by 20 cycles of 95 °C for 30s, 72 °C for 20 sec, and a final elongation at 72 °C for 5 min. Samples were kept at 4 °C. The Taq polymerase was inactivated by adding EDTA to 10mM and then incubated at 75 °C for 20 min. Five µl of the solution was removed to check on a gel by using 3% NuSeive / 1% Agarose in 1X TBE. A faint band at about 100bp was observed for the no enzyme control (ssDNA template), while a much brighter 130bp band than the ssDNA template was observed for the resulting annealed oligo. The RNA was synthesized by using the Maxiscript T7 Kit following the manufacture guides: 2µl dH2O, 10µl dsDNA template, 2µl 10X trans buffer, 1µl ATP, GTP,

UTP, CTP, respectively and 2µl Enzyme Mix were add per run. Samples were incubated at 37 °C for 1-2 hours, then mixed well with 1 µl of Turbo DNase I and incubated at 37 °C for 15 min. Five µl of 0.5M EDTA was added to chelate Mg ions. Turbo DNase I was heat inactivated at 75°C for 10 min. Purification of sgRNA using Zymo RNA clean and concentrator kit followed the manufacture's guideline. Phenol red was added to color the sgRNA/Cas9 solutions by mixing sgRNA, Cas9 protein and phenol red at a 1:1:1 ratio, this mixtures were then incubated for 10 minutes on ice before using.

Spawning

Sexually mature, P1 male channel catfish with delta-5 desaturase transgene, male channel catfish and female channel catfish that had not been fed for two days were harvested from at the Catfish Genetics Unit, EW Shell Research Center (Auburn University, AL). They were then acclimated to 25-27°C.

Female channel catfish were intraperitoneally injected with 90ug/kg BW luteinizing hormone-releasing hormone analog (LHRHa) implants and placed into labeled mesh bags in tanks with adequate water flow and oxygen (>5mg/L). After 36 hours, females were checked for eggs every 4 hours. If no eggs were seen on the bags after 100 hours, the females were re-injected with LHRHa. If eggs were found, the female was anesthetized with buffered 100ppm tricaine methane sulfonate (MS-222) solution, rinsed with fresh water, dried, and the eggs hand stripped into pie pans. To avoid sticking and clumping of the eggs and to facilitate hand stripping, Crisco was applied to both the female's genital opening and the pans. A 0.9% saline solution was used to rinse and remove blood from the eggs as needed.

Male channel catfish or P1 male channel catfish with delta-5 desaturase transgene were euthanized for removal of their testes in preparation for sperm extraction. The cleaned testes

were then macerated in a 0.9% saline solution at a ratio of 10ml saline to one gram of testes to release and dilute the sperm.

Microinjection

A total of 1-2ml of sperm solution from non-transgenic males was used to fertilize 200-300 eggs. Sperm was mixed with eggs gently in a spawning pan, and pond water (autoclave first) was added to slightly cover the eggs. After gently stirring the mixture for 30s, more pond water was added to allow the eggs to harden for 10-15mins.

A microneedle was opened by cutting slightly, then 8-10μl gRNA-Cas9 protein mixed solution was loaded into the needle by using micro-loader. The microneedle was attached to the micro-pipet holder, and then the holder was attached to the micro-manipulator. A nitrogen cylinder was opened to release the gas for microinjection, and the pressure was adjusted to ensure injection of approximately 40ng mixed solution per egg during injection. A single layer of eggs was placed on a clean 150 mm petri dish with a very thin layer of Crisco, then the petri dish was placed under the microscope and held with one hand and the needle was lowered with the other hand until it penetrated both the egg membrane and yolk sac. The mixed solution was microinjected into the yolk as close as possible to the blastodisc by pressing the pedal to apply pneumatic pressure. The injection material was spread throughout the yolk by withdrawing the needle while injecting. Then next egg was injected by retracting the needle smoothly and moving the petri dish gently. All the eggs were injected in one-cell stage.

F1 Generation

A total of 1-2ml of sperm solution from transgenic males was used to fertilize eggs from transgenic females. Sperm was mixed with eggs gently in a spawning pan, and pond water was added to slightly cover the eggs. After gently stirring the mixture for 30s, more pond water was

added to allow the eggs to harden for 10-15mins.

Embryo incubation and fish culture

After microinjection, approximately, 200-300 eggs were placed in a small plastic tub (10L) with Holtfreter's solution (59 mmol NaCl, 0.67 mmol KCl, 2.4 mmol NaHCO3, 0.76 mmol CaCl2, 1.67 mmol MgSO4) containing 10 ppm doxycycline with aeration (Armstrong et al., 1989; Bart & Dunham, 1996). Dead embryos were removed 2 times daily just prior to changing the Holtfreter's solution. After 5-7 days, fry were hatched and use of doxycycline ceased. Fry were fed with Artemia nauplii (San Francisco Bay Brand, Inc. Newark, CA) when the yolk was absorbed. Upon reaching swim up stage, larvae were fed with Purina® AquaMax® Fry Powder containing 50% crude protein three times a day to satiation. As fry grew larger they were stocked at 500 fry per 110L flow through tanks and fed with AquaMax® Fry Starter 100 containing 50% crude protein. After two months, fish were restocked at a rate of 150 fry per 110L flow through tank. As fish grew, feed was switched to AquaMax® Fry Starter 200 and then AquaMax® Fry Starter 300, containing 50% crude protein until fish were large enough to remove fin clips for PCR analysis. Then positive and negative individuals were pit tagged and stocked into 110L communal tanks at a density of 10 fish per tank and fed with AquaMax® Fry Starter 300 twice daily.

In the case of generation of F1 fully name the gene channel catfish, transgenic and non-transgenic eggs were fertilized with sperm from positive P1 desaturase males and allowed to water harden for one hour before eggs were moved to mesh egg baskets suspended in paddle-wheel troughs with adequate aeration until hatching and the yolk sac absorption. Once the fry in the fry reached the swim-up stage, the culture procedures were the same as the microinjected fry above. After 24 months, 37 fish including desaturase channel catfish and controls were weighed,

sexed and sacrificed to take muscle tissue sample.

DNA extraction and PCR

Individual fin clips were used for DNA extraction. Each fin sample was digested with 1.2 µl proteinase K with 600 µl cell lysis buffer, then incubated in a 55°C water bath for three hours and vortexed for a few seconds every hour. After digestion, a 170 µl protein precipitation solution was added per sample and vortexed for 20 seconds, then samples were kept on ice for 15 minutes. Then samples were centrifuged at 15000rcf for 10 minutes at room temperature. The supernatant was poured into a new tube and mixed with 600 µl isopropanol by inverting 20 times, followed by centrifugation at 15000rcf for 5 minutes at room temperature. Finally, 600 µl of 70% ethanol was used to wash the small pellet twice, and the DNA solution was adjusted with the addition of sterilized water until the concentration was approximately 500ng/µl.

PCR was used to identify transgenic fish by using the standard Platinum Taq (ThermoFisher Scientific Inc., Waltham, Ma) 10µl master mix, 2mM of dNTPs, 15mM MgCl2, and 10 units of Taq polymerase with 2mM of each primer D5D-3F (5'-CATCGTCACTTCCAGCACCA-3') and D5D-3R (5'-GCCACAGATCCCCTGACTTC-3') for the first round and primers D5D-2F (5'-GAAGTTGAAGTACATGCCCTACC-3') and D5D-2R (5'-GGTGTTCAATCTGAAAGTTGAGG-3') for the second round. The amplification procedure for both rounds was as follows: initial denaturation for 5 min at 94 °C, followed by 37 cycles of 94°C for 30s, 58°C for 30s and 72°C for 60s and the final elongation for 7 min at 72°C. Samples were then kept at 4°C. Finally, samples were run on a 1% agarose gel with ethidium bromide at 100 volts for 30 minutes. All PCR reactions had positive transgenic channel catfish, non-transgenic channel catfish and a blank control.

Lipid extraction and fatty acid analysis

Lipid extraction was accomplished following the protocol from Folch et al. (1957). Samples (with/without skin) were frozen with liquid nitrogen and then ground with a pestle and mortar.

Before extracting fatty acids, 50ml tubes and 50ml flasks were washed with pure water and dried (each sample had one repeat). Each flask was weighted and recorded. One g of tissue per muscle sample was weighted and mixed with 17ml of a chloroform/methanol (2:1) solution. Tissue samples were homogenized by using a handheld tissue homogenizer for a minute. The flask was put into the beaker flask. Then samples were poured into a stainless-steel vacuum filter (with glass micro fiber), and the water valve opened to apply suction with a vacuum tube to draw solvents from the sample through the filter. The filter piece was rinsed with the 2:1 chloroform solution to dislodge any lipid residues between samples. The solution volume was increased by adding 2:1 chloroform-methanol until the total volume was 20ml, then 4ml of 0.74% KCl solution was added to each flask. Each flask was flushed with N_2 for 5 to 8 seconds, then closed with a numbered cap and inverted twice. Samples were kept in the refrigerator overnight for phases separation.

Upper phase was removed with pipette and vacuum flask. A total of 3.5L deionized water was added to N-EVAP, the temperature was kept at 45°C and N_2 streams opened to allow for the evaporation of the remaining liquid in the flask. Once dry, each sample was weighted and recorded, the initial weight of each flask was then subtracted from the post extraction weight to calculate the lipid weight of each sample. Then 100 µl of internal standard (C 19:0, nonadecanoic acid methyl ester, Sigma- Aldrich, St. Louis, MO) and 1ml 0.5N KOH in MeOH to each sample (internal standard was prepared in hexane at a concentration of 0.04g/5ml hexane). Then, caps were placed back onto the flasks and samples placed in a 70°C water bath for 20min. After 20 minutes, flasks were removed from the water bath. 1ml BF3 in MeOH (14% BF3, Sigma-

Aldrich, St. Louis, MO) was added to flasks. Flasks were then flushed with N_2 and placed back into the 70°C water bath for 40 minutes. After 40 minutes, flasks were removed from the water bath and allowed to cool down to room temperature, 2ml hexane and 2ml saturated NaCl solution were added, and flasks were vortexed for 15s at the speed of 7 for phases separation. Then 22.86cm disposable Pasteur pipettes were used to transfer the upper layer (hexane layer) into numbered test tubes with glass fiber filters and a pinch of sodium sulfate. The remaining hexane in test tubes was then evaporated with N_2 and a calculated amount of hexane was added to samples to reach a concentration of 5mg lipid/ml of hexane.

Samples were mixed and then poured into injection vials that were labeled and stored at -20°C until they could be injected into a gas chromatograph (GC-MS6890). Gas chromatography-mass spectrometry of samples was performed using a Stabilwax-Da column (30m, 0.25mm ID, 0.25 μm) run on an Agilent 6890N gas chromatograph and detection accomplised with a 5875 mass spectrometer. Peaks were identified by comparing sample retention times to a standard mix (Supelco® 37 Component FAME Mix) and quantified using a C 19:0 nonadecanoate internal standard (Sigma-Aldrich Co., St.Louis, MO, USA). Oven temperature was initially set to 50^{o}C for 2 minutes, then increased to 183^{o}C at a rate of 50^{o}C per minute. The temperature was then increased to 230^{o}C at a rate of 3^{o}C per minute, where it was held for 1 minute. After detection with a GC-MS6890, data was analyzed by using Chrombox Q. FA levels were expressed as a percentage of total identified FAMES and on a per gram of wet tissue basis.

Proximate analysis

Proximate analysis was used to analyze fish muscle tissue components, including moisture, ash, protein, and fat. The method and results of fat extraction is the same as lipid extraction.

Moisture

Metal pans were labeled and placed in an oven (100°C) for 30 min, then removed and placed in a desiccator and allowed to cool. Approximately, 7.0g wet fish muscle was weighted, recorded and placed in pans (2 replicates per sample). Wet samples were dried in an oven (100°C) for 14-16h. Once dry, samples were placed in a desiccator to cool and the weight recorded. Moisture percentage was calculated as follows:

Moisture% = 100 - (weight of dry sample / weight of original sample)*100%.

Ash

Crucibles were labeled and placed in an oven (100°C) for 30 minutes, cooled in desiccator and weight recorded. Approximately, 1.0g dry sample was weighed, recorded and placed in crucibles (2 replicates per sample). The crucibles with samples were placed in a muffle furnace for 3-6 hours at 600°C, then placed in a desiccator and allowed to cool. Weight was recorded again and the ash percentage was calculated as follows:

Ash% = ash weight*100/ dry sample weight.

Protein

The protein content of dried sample was measured by the Kjeldahl technique (3 replicates per sample). A 10-15 mg sample of tissue was weighed, recorded and placed in a 30 ml digestion flask, and 0.4-0.6 g of catalyst was added to the digestion flask with two perforated boiling beads. Then, 3.0 ml of concentrated H_2SO_4 was added. Both hood exhaust and water aspirator were turned on. Flasks were placed on a digestion unit and the heating elements was set at 5.5. When samples turned clear green, the temperature was raised to 8 for exactly 20 minutes. Then flasks were removed and cooled to room temperature.

Distillation unit was activated by turning on the tap water to the condenser, and the heater element was set at 8. After the water in the outer chamber was boiling, the deionized water valve

was opened. Then, 10 ml of saturated boric acid was added to a 50 ml titration sample cup with a cocktail dispenser. After digestion flasks had cooled, approximately 4 ml of deionized water was slowly added to the sample cup and the contents were poured into the open sample cup on the still. The digestion flasks were rinsed with 2ml deionized water, and samples were washed into the inner chamber. The boiling beads were removed with forceps, followed by closing the sample addition valve on the still. The titration sample cup was placed under the condenser so that the tip of the condenser was submerged in the boric acid. Approximately, 10 ml of 40% NaOH from a medium beaker was added to the sample cup, NaOH was slowly dripped into the inner vessel until the solution turned brown. Then sample was distilled for exactly 5 minutes. The tip of the condenser was removed from the boric acid by lowering the sample cup and then the tip of the condenser rinsed by distilling for 1 more minute (Bradstreet, 1965; Ma & Zuazago, 1942; McKenzie & Wallace, 1954).

Samples were titrated and protein % calculated as follows:

% nitrogen = [(S-B)(14.007)(N)]/[Wt of sample (mg)] * 100

S = ml HCL to titrate the sample; B = ml HCL to titrate the blank; N = normality of HCL

% crude protein = % nitrogen * 6.25

Statistical Analysis

Statistical analysis was done by using R studio. A t-test was used to compare the results, and P values were calculated using a two-sample t-test. R code is shown in the appendix. Average coefficient of variation for body weight, fatty acid percentage, protein percentage, moisture percentage, fat percentage, ash percentage was calculated.

Results

Targeted gene insertion

Apo14-Masou salmon_Elovl2 gene was successfully inserted on chromosome 1 in one channel catfish utilizing microinjection and CRISPR/Cas9 mediated knockin (Figure4). A second individual was observed that had two different desaturase genes, Apo14-Rabbitfish_D4_desaturase gene and Apo14-Rabbitfish_D5/6_desaturase gene, inserted in the non-coding region of chromosome2 and chromosome7 respectively.

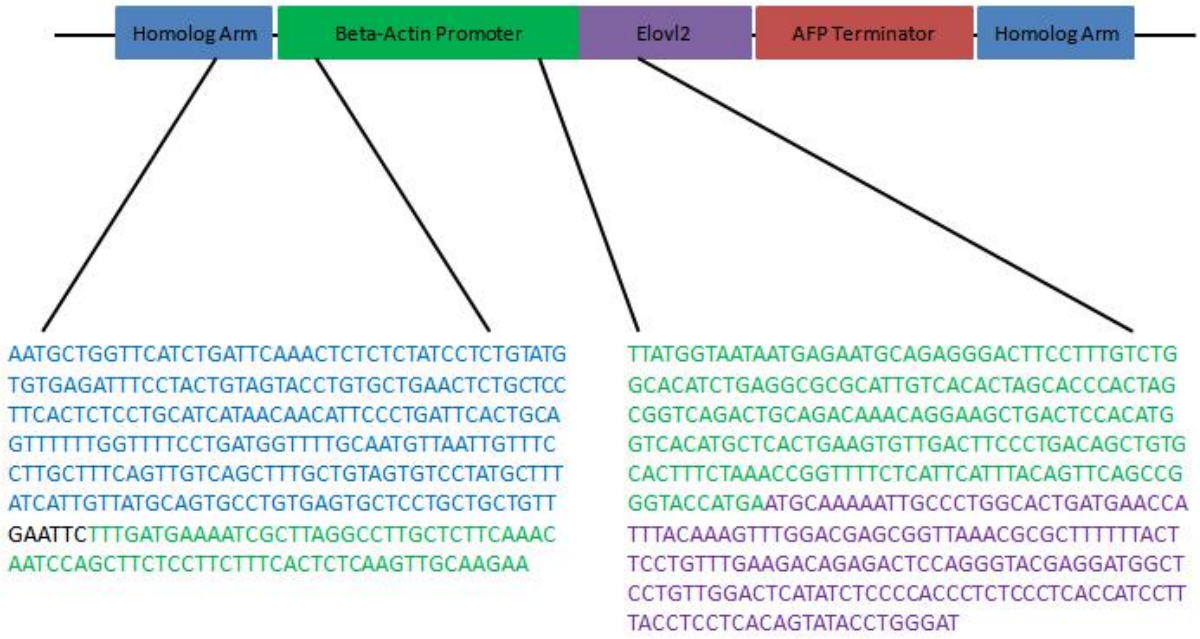

Figure 4: Sequencing result of Apo14-Masusalmon_Elovl2 gene on chromosome 1 in one channel catfish utilizing microinjection and CRISPR/Cas9 mediated knockin

Fatty acid composition

Precursors of long chain omega-3 fatty acids either did not change or decreased in transgenic fish compared to controls, as ALA was 5% ($P = 0.459$) lower as a percentage of total FAME in females and 19% ($P < 0.05$) lower in males, ETA was 7% ($P = 0.180$) higher in females and 3% ($P = 0.270$) lower in males. As the direct product of n-3 delta-5 desaturation, EPA was 23% ($P = 0.038$) higher as a percentage of total FAME in females and 30% ($P < 0.05$) higher in males. The two subsequent products were elevated as DPA increased by 16% ($P = 0.267$) and 27% ($P < 0.05$) in females and males, respectively, and DHA increased by 32% ($P = 0.024$) and 47% ($P < 0.05$) in females and males, respectively (Figure 5, 6, Table 1, 2).

Desaturase female fish had a lower CV for ALA, EPA, ETA, DHA and DPA than control females. Desaturase male fish had a lower CV for DPA percentage than control males, but higher CV in other fatty acid percentages (Table 3).

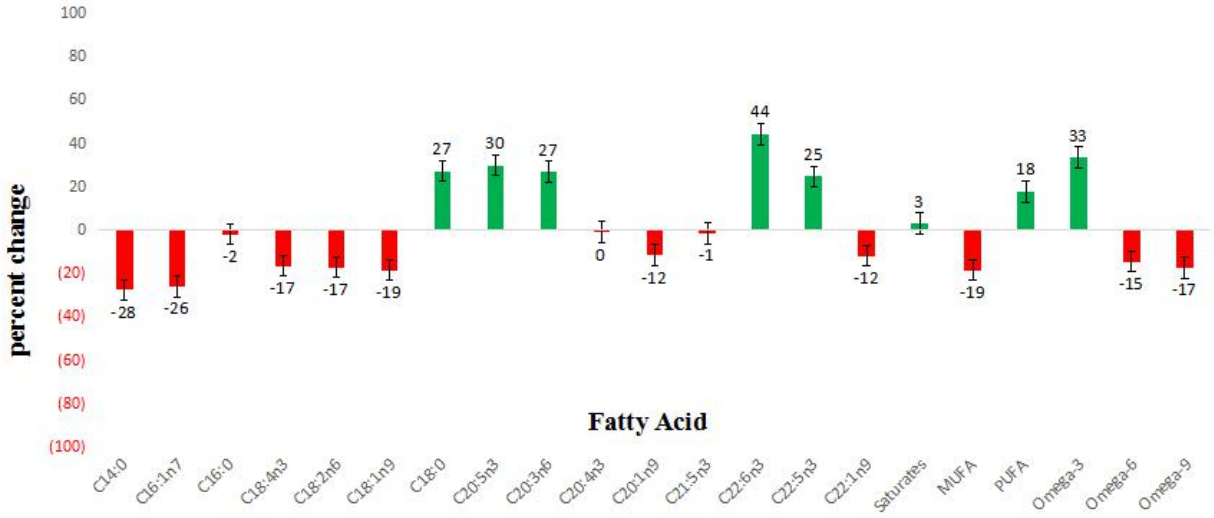

Figure 5: Percentage change in fatty acids in muscle of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5- deaturase transgenic channel catfish (2 females, 8 males, two replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families. Significant changes in fatty acid level were observed $\alpha = 0.05$.

Table 1: Mean mg/g weight (g weight of muscle sample) fatty acids for F1 male and female masou salmon (*Oncorhynchus masou*) delta 5- deaturase transgenic channel catfish (10 fish, two replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (27 fish) from 3 families.

mg/g Wet Weight	Control Channel Catfish	Transgenic Channel Catfish
C14:0	0.35	0.15**
C16:1n7	0.77	0.35**
C16:0	2.95	1.80**
C18:4n3 (ALA)	0.11	0.06**
C18:2n6 (LA)	1.73	0.88**
C18:1n9	4.48	2.26**
C18:0	1.01	0.80**
C20:5n3 (EPA)	1.11	0.90**
C20:3n6 (DGA)	0.11	0.09*
C20:4n3 (ETA)	0.15	0.09**
C20:1n9	0.62	0.33**
C21:5n3	0.04	0.03**
C22:6n3 (DHA)	1.79	1.63
C22:5n3 (DPA)	0.45	0.36**
C22:1n9	0.23	0.12*
Saturates	4.30	2.75**
MUFA	6.10	3.07**
PUFA	5.49	4.03**
Omega-3	3.65	3.06*
Omega-6	1.84	0.97**
Omega-9	5.33	2.71**
total	15.89	9.85**

Totals calculated as follows: *Significant at $P < 0.05$; **Significant at $P < 0.01$

Saturates: 14:0, 16:0, 18:0 Internal standard 19:0 not included.

MUFA: 16:1n7, 18:1n9, 20:1n9, 22:1n9

PUFA: 18:4n3, 18:2n6, 20:5n3, 20:3n6, 20:4n3, 21:5n3, 22:6n3, 22:5n3

Total n-3: 18:4n-3, 20:5n-3, 20:4n3, 21:5n3, 22:6n-3, 22:5n3

Total n-6: 18:2n-6, 20:3n-6

Total n-9: 18:1n9, 20:1n-9, 22:1n9

Table 2: Mean percent fatty acid methyl ester (FAME) for F1 channel catfish, *Ictalurus punctatus*, transgenic for delta-5 desaturase compared to control channel catfish.

FAME %	Control Channel Catfish	Transgenic Channel Catfish
C14:0	2.12	1.53**
C16:1n7	4.75	3.50**
C16:0	18.63	18.25
C18:4n3 (ALA)	0.68	0.57**
C18:2n6 (LA)	10.66	8.82**
C18:1n9	27.82	22.67**
C18:0	6.49	8.25**
C20:5n3 (EPA)	7.13	9.25**
C20:3n6 (DGA)	0.69	0.88**
C20:4n3 (ETA)	0.95	0.95
C20:1n9	3.81	3.37*
C21:5n3	0.27	0.27
C22:6n3 (DHA)	11.64	16.77**
C22:5n3 (DPA)	2.93	3.66**
C22:1n9	1.42	1.25
Saturates	27.24	28.04
MUFA	37.80	30.79**
PUFA	34.96	41.16**
Omega-3	23.61	31.46**
Omega-6	11.35	9.70**
Omega-9	33.05	27.29**

Totals calculated as follows:

Saturates: 14:0, 16:0, 18:0 Internal standard 19:0 not included.

MUFA: 16:1n7, 18:1n9, 20:1n9, 22:1n9

PUFA: 18:4n3, 18:2n6, 20:5n3, 20:3n6, 20:4n3, 21:5n3, 22:6n3, 22:5n3

Total n-3: 18:4n-3, 20:5n-3, 20:4n3, 21:5n3, 22:6n-3, 22:5n3

Total n-6: 18:2n-6, 20:3n-6

Total n-9: 18:1n9, 20:1n-9, 22:1n9

*Significant at $P < 0.05$

**Significant at $P < 0.01$

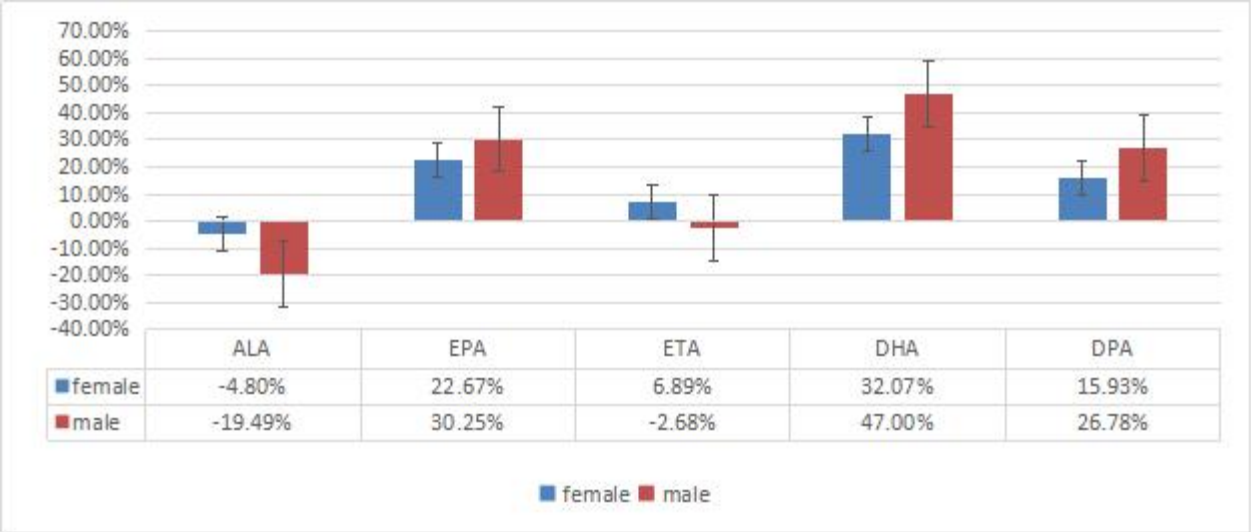

Figure 6: Percentage change in n-3 fatty acids in muscle of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5- deaturase transgenic channel catfish (2 females, 8 males, two replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families. No significant changes in n-3 fatty acid level were observed $\alpha = 0.05$.

Table 3: CV of ALA, EPA, ETA, DHA and DPA of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5- deaturase transgenic channel catfish (2 females, 8 males, two replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families.

CV	ALA	EPA	ETA	DHA	DPA
control female	30.71%	32.32%	30.79%	33.69%	33.81%
treatment female	9.33%	0.73%	1.64%	1.86%	0.42%
control male	8.30%	9.59%	6.79%	13.39%	12.02%
treatment male	14.40%	12.00%	8.81%	13.64%	6.49%

ALA: α-linolenic Acid

DHA: Docosahexaenoic Acid

DPA: Docosapentaenoic Acid

EPA: Eicosapentaenoic Acid

ETA: Eicosatetraenoic Acid

Omega-3 fatty acid production.

The omega-3 fatty acid production percentage was not different (P = 0.319) between males and females in the transgenic group, as well as in the control group (P =0.844). The omega-3 fatty acid production percentage of F1 desaturase female channel catfish was 24.94% higher than controls (P = 0.026), while the n-3 fat percentage of male desaturase channel catfish was 34.87% higher than that of control males (P < 0.0001) (Figure 7).

Mean coefficient of variation for omega-3 fatty acid production, as a on percentage basis, for desaturase female fish was 1.52% while control females showed increased variation with a CV of 12.53%. Desaturase male fish had a mean CV for omega-3 fatty acid production percentage of 11.09%, while control males had a CV of 10.23%. The variance of Omega-3 fatty acid production percentage of male and females between desaturase and controls were not significant different (P = 0.201, P = 0.249) respectively.

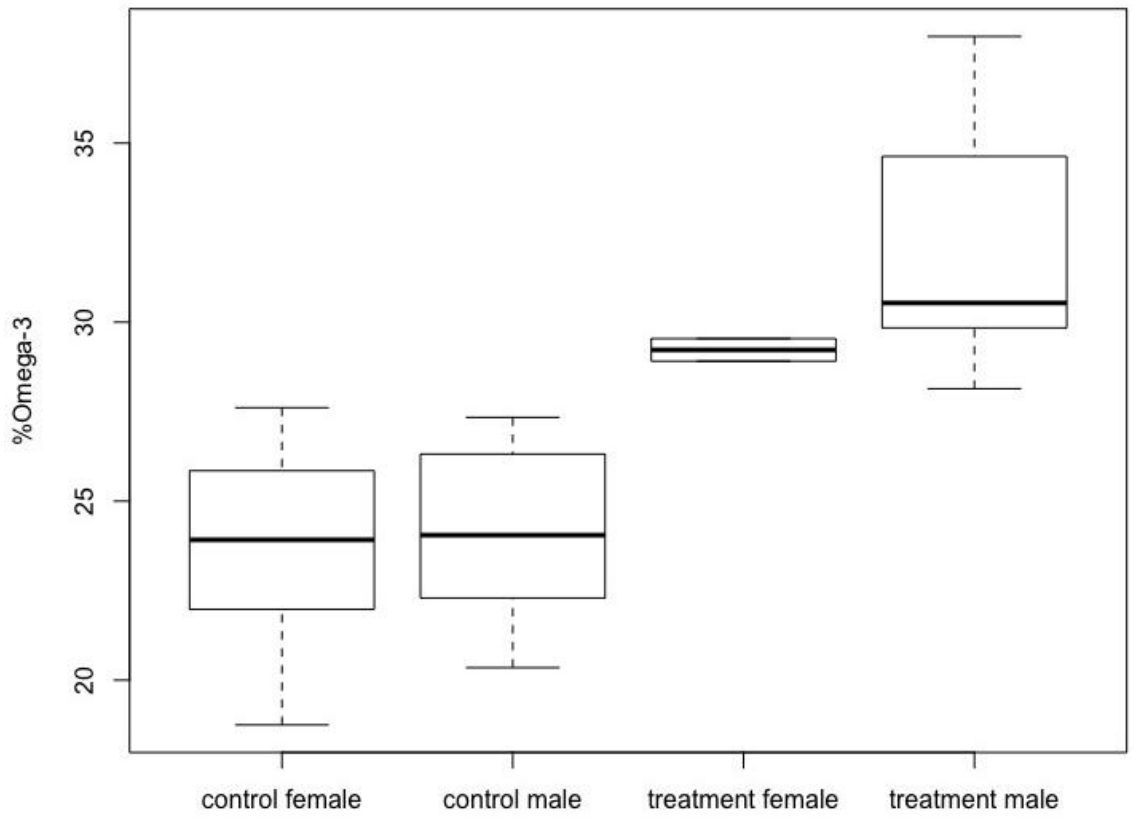

Figure 7: Omega-3 fatty acid production percentage of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5- deaturase transgenic channel catfish (2 females, 8 males, two replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families. Significant changes in n-3 fatty acid level were observed $\alpha = 0.05$ ($P < 0.05$, $P = 0.026$).

Omega-6 fatty acid production in transgenic fish.

The Omega-6 fatty acid production percentage of males and females were not significant different in the transgenic group (P = 0.379), as well as in the control group (P = 0.243). The Omega-6 fatty acid production percentage of F1 desaturase female channel catfish was 6.32% lower than that of controls, but not significant (P = 0.398), while the n-6 fat percentage of male desaturase channel catfish was 17.54% lower than that of control males (P = 0.001) (Figure 8).

Mean coefficient of variation for omega-6 fatty acid production, as a on percentage basis, for desaturase female fish was 1.27% while control females showed increased variation with a CV of 8.66%. Desaturase male fish had a mean CV for omega-3 fatty acid production percentage of 10.85%, while control males had a CV of 10.29%. The variance of Omega-6 fatty acid production percentage of male and females between desaturase and controls were not significant different (P = 0.628, P = 0.208) respectively.

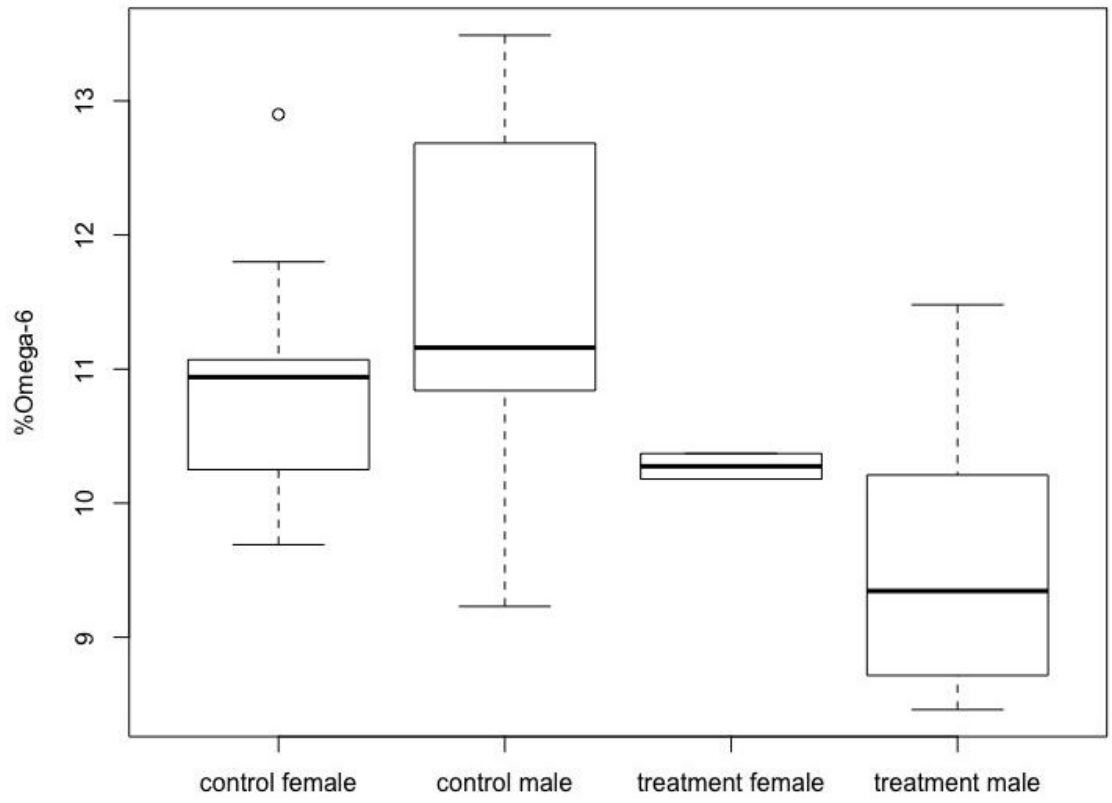

Figure 8: Omega-6 fatty acid production percentage of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5- deaturase transgenic channel catfish (2 females, 8 males, two replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families. Significant changes in n-3 fatty acid level were observed $\alpha = 0.05$ ($P = 0.001$, $P = 0.398$).

Skin effects on n-3/n-6 fatty acid samples from controls

Samples tested with skin on had different levels of n-3/n-6 fatty acids compared to samples without skin (Figure 9). The n-3 fatty acid level and n-6 fatty acid level of channel catfish samples with skin on and without skin were different in the male group, as well as the female group. The n-3 fatty acid level of female channel catfish samples without skin was 41.18% higher than that of samples with skin ($P = 0.052$), and the males group was 35.33% higher ($P = 0.002$). The n-6 fatty acid level of female channel catfish samples without skin was 26.82% lower than that of samples with skin ($P = 0.019$), and the male group was 20.86% lower ($P = 0.006$).

Precursors of the long chain n-3 fatty acids in samples from females without skin, such as ALA were 8.17% ($P = 0.116$) lower as a percentage of total FAME than samples with skin, ETA was 7.81% ($P = 0.101$) higher without skin. As the direct product of n-3 delta-5 desaturation, EPA was 29.43% ($P = 0.072$) higher as a percentage of total FAME in females without skin. Another two subsequent products DPA was 38.70% higher ($P = 0.067$) and DHA was 62.39% higher ($P = 0.056$) without skin.

Precursors of the long chain n-3 fatty acids in samples from males without skin, such as ALA were 5.45% ($P = 0.670$) lower as a percentage of total FAME than samples with skin, ETA was 8.18% ($P = 0.024$) higher without skin. As the direct product of n-3 delta-5 desaturation, EPA was 31.11% ($P = 0.003$) higher as a percentage of total FAME in females without skin. Another two subsequent products DPA was 25.93% higher ($P < 0.001$) and DHA was 47.74% higher ($P = 0.002$) without skin.

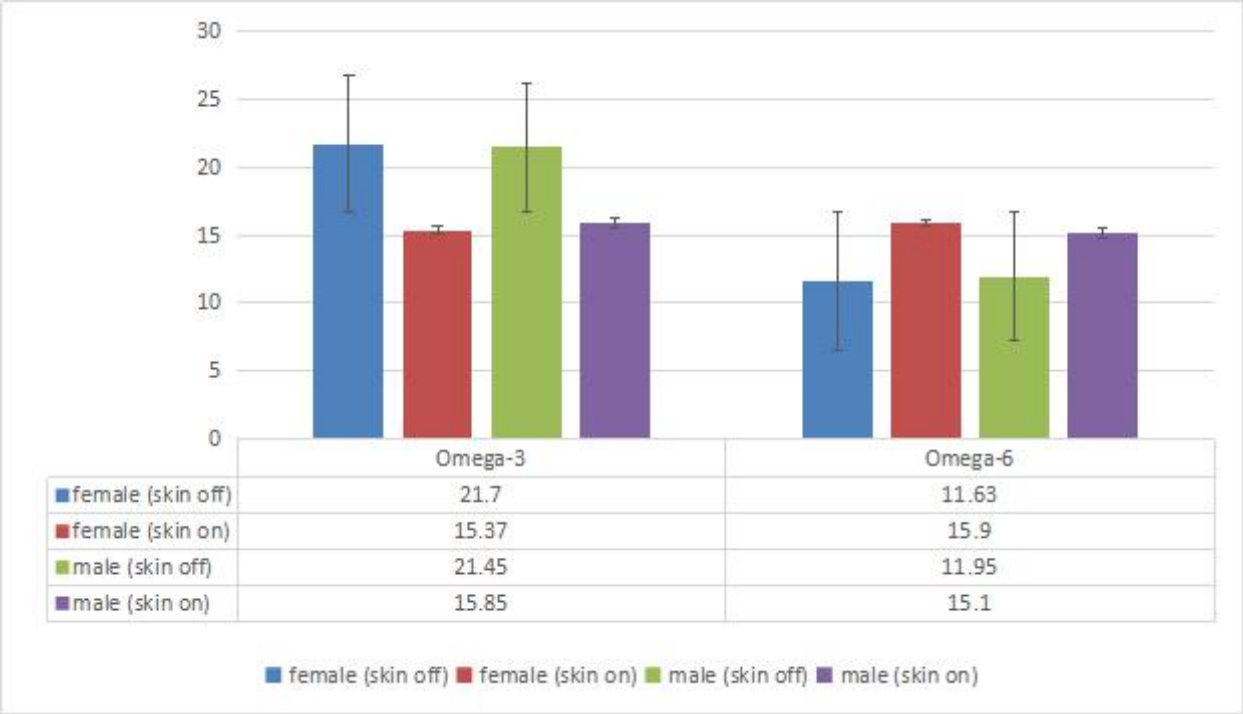

Figure 9: Percentage change in N-3/N-6 fatty acids in muscle samples with skin of control F1 male and female channel catfish, *Ictalurus punctatus,* grown in flow through tanks relative to samples without skin (3 females, 4 males, two replicates per fish). Significant changes in N-3/N-6 fatty acids level were observed $\alpha = 0.05$.

Fat percentage (wet weight)

The fat percentage of males and females was different within the transgenic group ($P = 0.065$), as well as in the control group ($P = 0.655$). Overall, the mean fat percentage of males in the control group and genetic group were higher than that of females, but the difference was not significant. The fat percentage between F1 desaturase female channel catfish and controls was not different ($P = 0.350$), but the fat percentage of male desaturase channel catfish was 42.84% lower than control males ($P < 0.001$) (Figure 10).

Desaturase female channel catfish had a mean CV for fat percentage of 14.50% while control females showed increased variation with a CV of 34.44%. Desaturase male channel catfish had a mean CV of 13.98% while control males showed increased variation with a CV of 25.25%. The variance of the fat percentage of the transgenic individuals was lower than that of the controls (Table 4). The variance of fat percentage of male and females between desaturase and controls were not significant different ($P = 0.005$, $P = 0.482$).

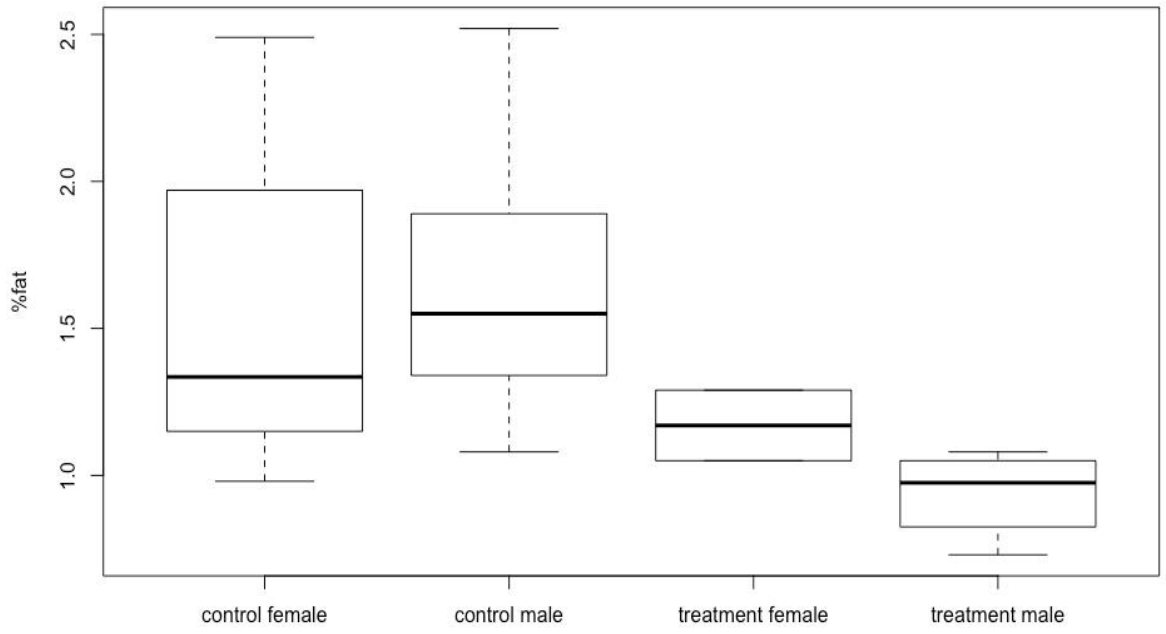

Figure 10: Fat percentage of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5-deaturase transgenic channel catfish (2 females, 8 males, two replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families. Significant changes in fat percentage were observed $\alpha = 0.05$ ($P < 0.001$, $P = 0.350$).

Table 4: Mean, SD, CV of fat percentage of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5- deaturase transgenic channel catfish (2 females, 8 males, two replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families.

		Mean (%)		SD (%)		CV	
Control (n=27)	Female (n = 10)	1.61	1.56	0.46	0.54	28.31%	34.44%
	Male (n = 17)		1.64		0.41		25.25%
Positive (n = 10)	Female (n = 2)	0.98	1.17	0.16	0.17	16.38%	14.50%
	Male (n = 8)		0.94		0.13		13.98%

Protein percentage (wet weight)

The protein percentage of males and females was not different in the transgenic group ($P = 0.161$), as well as in the control group ($P = 0.613$). Overall, the mean protein percentage for pooled males was lower than that of females, but the difference was not significant. The protein percentage of F1 desaturase female channel catfish was 40.23% higher than controls ($P = 0.115$), and the protein percentage of male desaturase channel catfish was 33.67% higher than control males ($P < 0.001$) (Figure 11).

Desaturase female fish had a CV of 6.89% and control females 27.85%. Desaturase male fish had a CV of 8.94% while control males showed increased variation with a CV of 11.75%. Overall, the variance of the protein percentage of the controls was higher than that of the transgenic individuals (Table 5). The variance of protein percentage of female and males between desaturase and controls were not significant different ($P = 0.506$, $P = 0.808$).

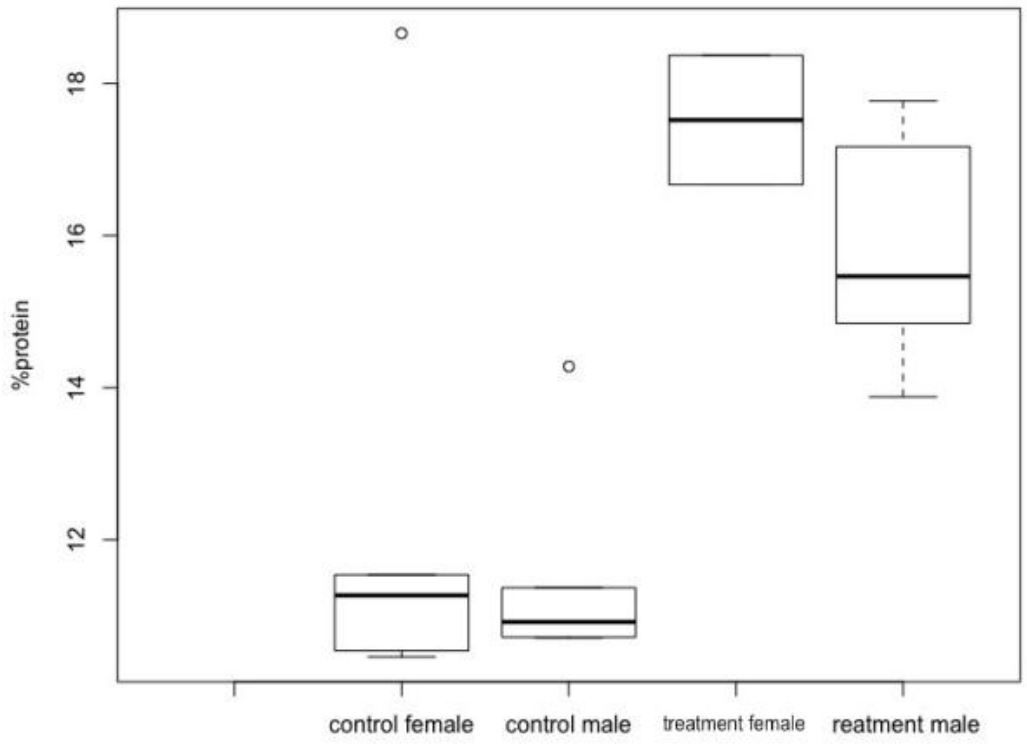

Figure 11: Protein percentage of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5- deaturase transgenic channel catfish (2 females, 8 males, three replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families. Significant changes in protein percentage were observed $\alpha = 0.05$ ($P < 0.001$, $P = 0.115$).

Table 5: Mean, SD, CV of protein percentage of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5- deaturase transgenic channel catfish (2 females, 8 males, three replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families.

		Mean (%)		SD (%)		CV	
Control (n=13)	Female (n = 5)	12.09	12.50%	2.30	3.48%	19.00%	27.85%
	Male (n = 8)		11.84%		1.39%		11.75%
Positive (n = 10)	Female (n = 2)	16.17	17.52%	1.49	1.21%	9.24%	6.89%
	Male (n = 8)		15.83%		1.42%		8.94%

Ash percentage (dry weight)

The percent ash of transgenic desaturase males and females was not different in the transgenic group (P = 0.58), and in the control group (P = 0.35) were higher than that of females, but the difference was not significant. The ash percentage difference between F1 desaturase female channel catfish and controls was not significant (P = 0.87), as well as between F1 desaturase males and controls (P = 0.79). The ash percentage of F1 desaturase channel catfish and controls were not different (Figure 12).

Desaturase female fish had an average CV of 2.5% while control females showed increased variation with a CV of 8.9%. Desaturase male fish had an average variation in ash percentage of 8.9% while control males showed increased variation with a CV of 10.7%. The variance of the ash percentage of the controls was higher than that of the transgenic individuals. (Table 6). The variance of percent ash of female and males between desaturase and controls were not significant different (P = 0.447, P = 0.675).

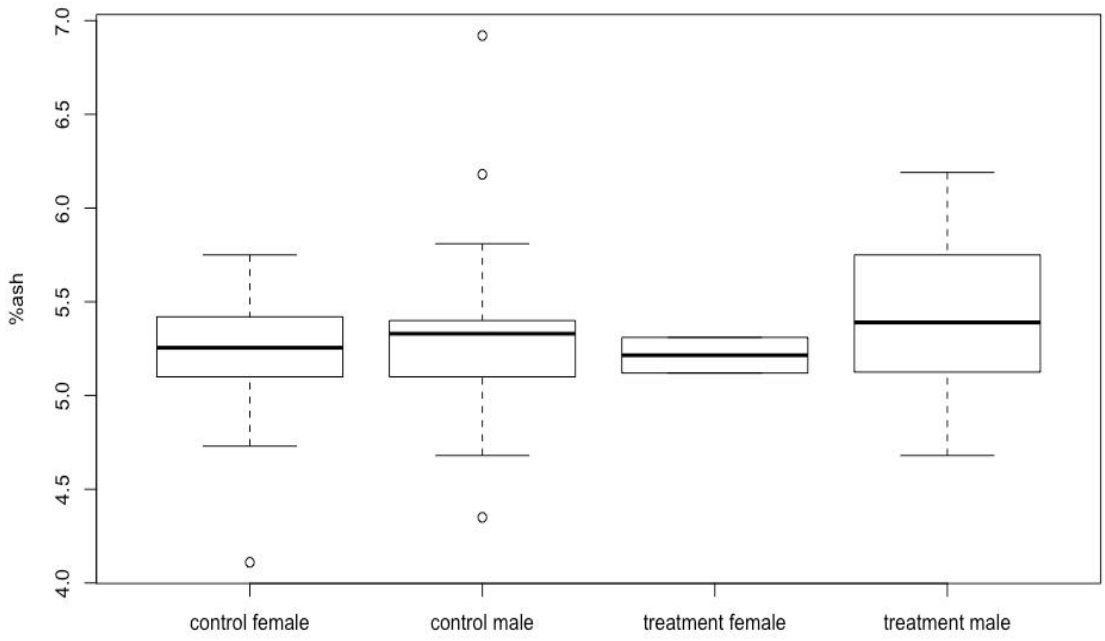

Figure 12: Ash percentage of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5- deaturase transgenic channel catfish (2 females, 8 males, two replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families. No significant changes in ash percentage were observed $\alpha = 0.05$ ($P = 0.79$, $P = 0.87$).

Table 6: Mean, SD, CV of ash percentage of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5- deaturase transgenic channel catfish (2 females, 8 males, two replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families.

		Mean (%)		SD (%)		CV	
Control (n=27)	Female (n = 10)	5.471	5.16	1.27	0.46	23.13%	8.91%
	Male (n = 17)		5.36		0.57		10.71%
Positive (n = 10)	Female (n = 2)	5.381	5.21	0.44	0.13	8.15%	2.54%
	Male (n = 8)		5.42		0.48		8.94%

Moisture percentage (wet weight)

The moisture percentage between males and females showed no significant difference in the transgenic group (P = 0.112), as well as in the control group (P = 0.258). The overall mean moisture percentage of males in the control group and transgenic group were higher than that of females, but the difference was not significant. The moisture percentage between F1 desaturase female channel catfish and female controls was not significant (P = 0.911), but the moisture percentage of male desaturase channel catfish was higher than controls (P = 0.002). However, when sexes were pooled, the average moisture percentage of desaturase channel catfish and controls was not different (Figure 13).

Desaturase female fish had a CV for moisture percentage of 1.54% while control females showed increased variation with a CV of 2.49%. Desaturase male fish had a CV for moisture percentage of 1.80% while control males showed decreased variation with a CV of 1.06% (Table 7). The variance of moisture percentage of male and females between desaturase and controls were not significant different (P = 0.064, P = 0.901).

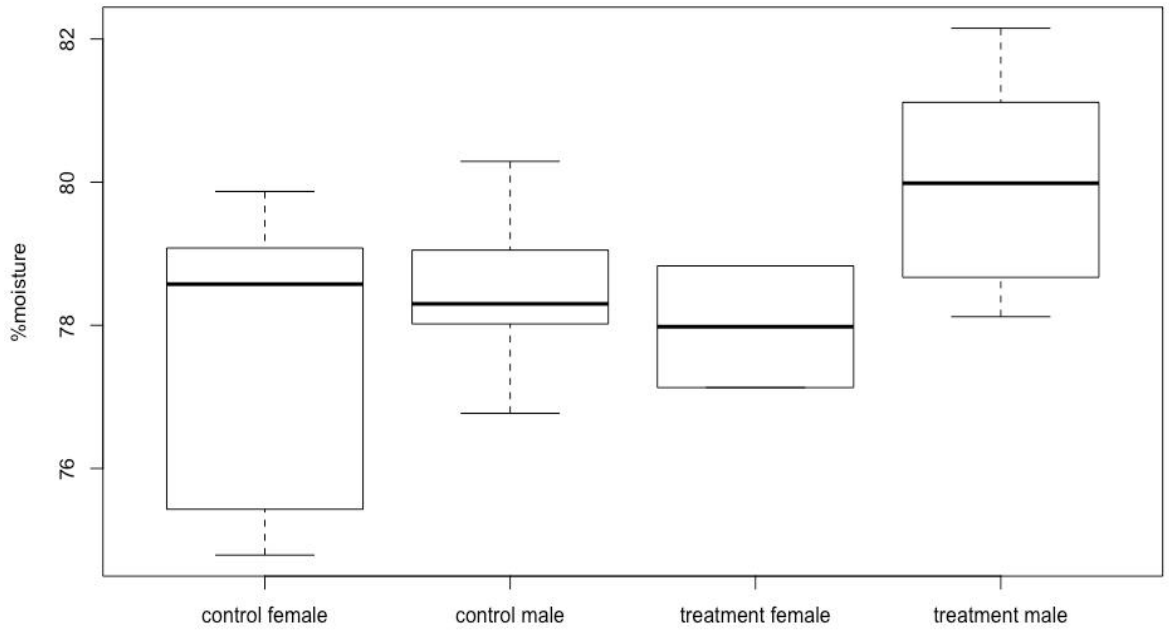

Figure 13: Moisture percentage of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5- deaturase transgenic channel catfish (2 females, 8 males, two replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families. No significant changes in moisture percentage were observed $\alpha = 0.05$ ($P = 0.002$, $P = 0.911$).

Table 7: Mean, SD, CV of moisture percentage of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5- deaturase transgenic channel catfish (2 females, 8 males, two replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families.

		Mean (%)		SD (%)		CV	
Control (n=27)	Female (n = 10)	78.20	77.81	1.35	1.93	1.72%	2.49%
	Male (n = 17)		78.43		0.83		1.06%
Positive (n = 10)	Female (n = 2)	79.58	77.98	1.58	1.20	1.98%	1.54%
	Male (n = 8)		79.98		1.44		1.80%

Body weight

The body weight of males and females were not significant different in the transgenic group (P = 0.324), as well as in the control group (P = 0.617). The body weight was not different between F1 desaturase female channel catfish and female controls (P = 0.785), but the body weight of male desaturase channel catfish was higher than that of controls (P = 0.012). When sexes were pooled, the mean body weight of F1 male and female desaturase channel catfish was higher than controls (Figure 14).

Desaturase female fish had an average CV for body weight of 6.4% while control females showed increased variation with a CV of 32.4%. Desaturase male fish had a CV for body weight of 17.8% while control males had a CV of 28.3% (Table 8). The variance of body weight of male and females between desaturase and controls were not significantly different (P = 0.638, P = 0.323).

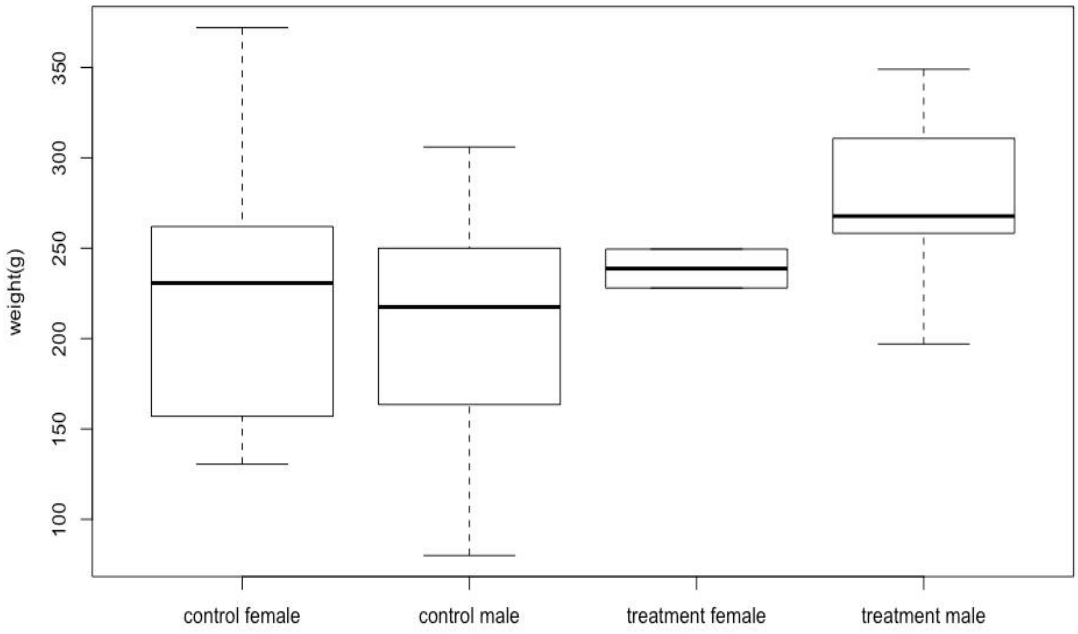

Figure 14: Body weight of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5-deaturase transgenic channel catfish (2 females, 8 males), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families. Significant changes in body weight were observed $\alpha = 0.05$ ($P = 0.012$, $P = 0.785$).

Table 8: Mean, SD, CV of body weight of F1 male and female masou salmon (*Oncorhynchus masou*) delta 5- deaturase transgenic channel catfish (2 females, 8 males), *Ictalurus punctatus,* grown in flow through tanks relative to controls (10 females, 17 males) from 3 families.

		Mean (g)		SD (g)		CV	
Control (n=27)	Female (n = 10)	215.59	223.80	63.65	72.58	29.52%	32.43%
	Male (n = 17)		210.76		59.60		28.28%
Positive (n = 10)	Female (n = 2)	269.70	238.75	46.83	15.20	17.36%	6.37%
	Male (n = 8)		277.44		49.44		17.82%

Tolerance of Low DO and Disease Resistance

One year post hatch there was an oxygen depletion in a communal tank containing transgenic desaturase channel catfish fingerlings and negative control full-siblings. The population within this tank was 20.0% transgenic. The observed percentage of transgenic fish among the mortalities was 7.32%.

One year post hatch there was a mortality event in a communal tank containing both transgenic desaturase channel catfish and negative control full-siblings suspected to be caused by disease (*columnaris*). The population of this tank was 58.33% transgenic but no transgenic fish was detected.

Family variation

Fish from different families had different level of protein percentage, but the ash, moisture and fat percentage were not different (Table 9). The protein percentage of fish from family 3 was 34.87% higher than that of family 1 ($P = 0.008$) and 27.39% higher than that of family 2 ($P = 0.010$). The protein percentage of fish from family 2 also was 5.87% higher than that of family 1 ($P = 0.015$) (Figure 15). The variance between protein percentage of family1 and family2 was not significant different ($P = 0.917$), but the variance of protein percentage of family3 was higher than that of family1 and family 2 ($P = 0.03$, $P = 0.02$) (Figure 15).

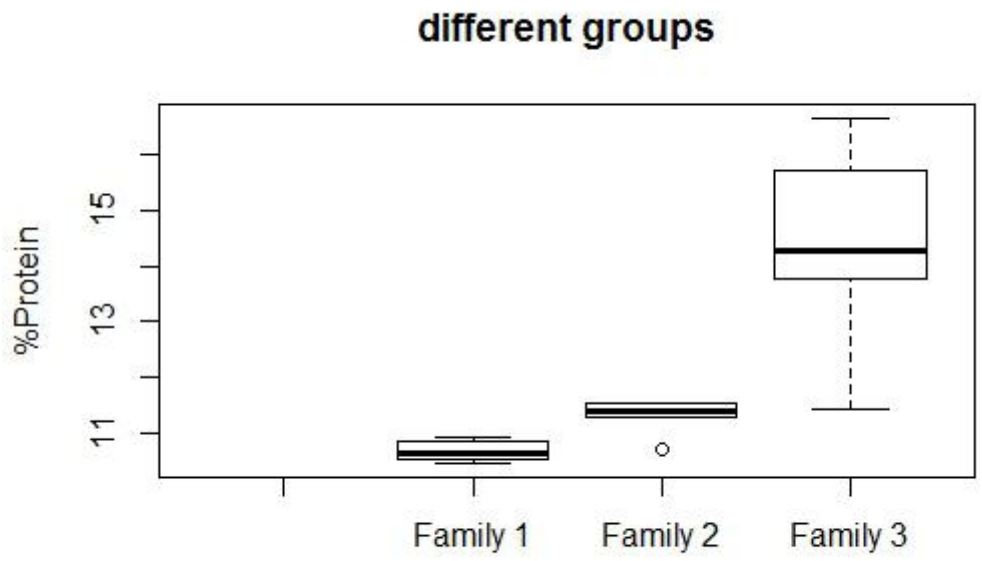

Figure 15: Protein percentage of channel catfish (n=14), *Ictalurus punctatus*, grown in flow through tanks from 3 different families. Significant changes in protein percentage were observed $\alpha = 0.05$ ($P_{family1,3} = 0.008$, $P_{family2,3} = 0.010$, $P_{family1,2} = 0.015$).

Table 9: Mean n-3%, fat%, protein%, ash%, body weight and moisture% of F1 transgenic and control channel catfish, *Ictalurus punctatus*, grown in flow through tanks from 3 families.

Family		N-3%	Fat%	Protein%	Ash%	Weight(g)	Moisture%
1	transgenic	30.2	1.04	14.47	5.39	232.75	81.11
	control	23.0	1.59	10.66	5.26	226.19	78.10
2	transgenic	31.7	1.01	16.78	5.23	261.88	79.04
	control	24.1	1.56	11.28	5.34	173.88	78.85
3	transgenic	32.2	0.94	16.40	5.53	296.00	79.35
	control	24.1	1.71	14.38	5.14	248.25	77.53

Discussion

CRISPR/Cas9 mediated knock-in using homology driven repair has revolutionized the ability to precisely insert transgenes in a variety of organisms including many model organisms, mice (Chu et al., 2016), fruit flies (Xu et al., 2014) and zebrafish, *Danio rerio* (Auer et al., 2014) and even more recently in aquaculture species such as rohu carp, *Labeo rohita* (Chakrapani et al., 2016). In this study, a novel approach takes this one step further through the targeted insertion of multiple transgenes to alter multiple steps in a pathway, which has not been previously reported in aquaculture species. Apo14-Masu salmon_Elovl2 gene was successfully inserted on chromosome 1 in one channel catfish utilizing microinjection and CRISPR/Cas9 mediated knockin. Additionally, CRISPR/Cas9 mediated knock-in resulted in the simultaneous insertion of two different desaturase genes into two non-coding regions of two different chromosomes of channel catfish through microinjection of eggs. Placing transgenes in non-coding regions should prevent or at least reduce off-target effects of insertions, which in turn might turn reduce negative effects on the individual. This method also increases the chance of producing homologous insertion and reduces the time it will take to produce a homozygous line while avoiding the need for inbreeding.

A masou salmon delta-5 desaturase transgene previously transferred to channel catfish altered n-3 fatty acid production. Body composition was altered, pleiotropic effects on performance traits were observed and the transgene appeared to increase uniformity of these traits.

The salmon desaturase gene actually decreased the absolute quantity of n-3 fatty acids in transgenic channel catfish compared to full-sibling controls, however, the fatty acid profile was changed such that the percentage of n-3 fatty acids based upon total fatty acids increased by 50%.

Thus, consumption of these fish would not increase a person's total intake of n-3 fatty acids, but the balance of the fatty acids would be a healthier ratio. Although sample size was small, the increased % of n-3 fatty acids was more dramatic in males than females.

Not surprisingly, the increased percentage of the desirable n-3 fatty acids and n-3 fatty acids at the end of pathway, EPA, DPA and DHA, led to changes in the levels of the precursors in the fatty acid pathway. The observed percentages of ALA, ETA were decreased in transgenic fish, and as predicted by the greater increase in n-3 fatty acids in males compared to females, the decrease in precursors were, in general, also greater in males. Utilizing desaturase transgenesis fish has the potential to improve valuable fatty acid profiles in farmed freshwater fishes that are normally low in n-3 fatty acid or that do not have the healthiest ratios of fatty acids in their muscle.

The n-3 fatty acid level increases obtained in the current experiment (1.25-fold and 1.34-fold of females and males, respectively) were similar (1.11-fold) to additional F1 delta 5 desaturase transgenic channel catfish evaluated by (Bugg, 2017). However, the channel catfish results were different from those found in zebrafish, delta 5 desaturase transgenic F1 and F2 zebrafish showed 21% and 24% increased levels of EPA and DHA content (Alimuddin et al., 2007). However, transgenes may not always get same effect in different species, variation may be related to position effect, copy numbers, and genetic background (Cheng et al., 2010; Shawi, Kinnaird, Burke & Bishop, 1990; Hobbs, Warkentin & DeLong, 1993; Logue, Owen, Rasmussen & Wehner, 1997; Scott, Woodfield & White, 1998).

Depending upon the culture, species and method of cooking, fish can be eaten with and without the skin of the flesh or muscle. Thus, any differences in fatty acid levels in various tissues could impact conclusions regarding the nutritional value of the transgenic product.

Additionally, sampling of different tissues may be desirable to implement non-lethal testing or different tissues may vary in ease of preparation for analysis. Thus, again, variation among tissues for fatty acid level could impact the usefulness of different types of non-lethal sampling. In the current study, the percentage of n-3 fatty acids in relation to total fatty acids was higher in muscle than in skin. This is a positive outcome as traditionally catfish are processed and eaten with the skin removed in the US.

Unexpectedly, male transgenic desaturase channel catfish had a 42.8% reduction in total fat compared to controls. The mechanism of converting precursors to longer chain fatty acids is perhaps inefficient leading to a reduction of total fat (Das, 2006; Jones et al., 2014; Harris et al., 2010). This is the likely reason that the desaturase transgenesis results in muscle with a healthier n-3 fatty acid profile, but an overall reduction in n-3 fatty acids. Since increased totals of n-3 fatty acids is a goal, this shortcoming might be addressed by selection, altering the diet as it influences the levels of n-3 fatty acids generated (Cheng et al. 2014) or by altering other steps in the fatty acid pathway with additional transgenes. The level of total fat is also important as it can influence flavor, texture and storage of processed product (Guichard, 2002; Piccinali & Sigrist, 1996; Ventanas et al., 2000; Pereira et al., 2006). The difference in fatty acid composition between males and females may be due to changes in hormone levels during gonad development.

Fat % and protein % are usually diametrically opposed in muscle. This is the case in growth hormone gene transgenic mammals and fish. The protein of transgenic carp muscle (19%) was higher than the control carp muscle (18%), but mean total fatty acid for these two group was not different (Chatakondi et al., 1995). Transgenic G119K and M4 mice had decreased body protein percentages and increased body fat percentages (Knapp et al., 1994). Transgenic common carp muscle had lower moisture percentages, higher protein content in both F1 and F2 generations

(Dunham et al., 2002). This was the result with the desaturase transgenic channel catfish in the current study. The protein % in the muscle was increased by approximately 40% in the transgenic individuals compared to full-sibling, non-transgenic controls. Again, this has implications for both nutritional value of the transgenic individuals as well as flavor since the protein to fat ratio is altered, total amino acids are increased with amino acid quantity and ratios influencing the detection of taste for the human palate (Colmenero et al., 1995).

Moisture % also likely affects flavor, texture and storage of flesh. In this case, desaturase transgenic males had higher percent moisture compared to full-sibling controls, some previous studies had similar results as moisture % of transgenic salmon was 5–6% greater than non-transgenic controls (Cook et al., 2000). When we look at the overall body composition changes of desaturase transgenic channel catfish, it is substantially different than non-transgenic channel catfish. Since this could influence texture, flavor and storage time, these parameters should be evaluated for desaturase channel catfish.

Pleiotropic effects and sex effects were apparent for several performance traits of desaturase channel catfish. The observed body weight was 6.7% higher in transgenic females and 31.7% higher in transgenic males compared to non-transgenic, full-sibling controls. In some manner, the functioning of the fatty acid pathway or the desaturase enzymes have an impact on growth that is sex related. In this case, a positive pleiotropic effect results in enhanced growth rate. Previous research also found that the male transgenic zebrafish fed a commercial diet and *Artemia* nauplii was 1.18-fold higher than its corresponding nontransgenic male fish, while fish fed only *Artemia* was 1.06-fold and 1.15-fold higher (Alimuddin, 2007).

An additional positive pleiotropic effect was observed for tolerance of low oxygen. One year post hatch there was an oxygen depletion in a communal tank containing transgenic

desaturase channel catfish fingerlings and negative control full-siblings. The survival of the transgenic individuals was 3-fold higher than controls. However, not all pleiotropic effects on survival traits were positive. When a low level columnaris epizootic occurred in two flow-through tanks containing desaturase transgenic fingerlings, the first 8-10 fish and the only fish to die were transgenic for desaturase. Challenges need to be conducted to determine the resistance of desaturase transgenic channel catfish during more severe outbreaks of columnaris as this is a critical trait impacting their value under commercial aquaculture conditions.

Another unusual result focused on the phenotypic variability of the desaturase transgenic channel catfish. The CV for virtually all of the traits measured, n-3 fatty acid percentage, body composition and growth rate, was substantially lower for transgenic individuals compared to their full-sibling controls. The mechanism for this transgene promoting uniform phenotypes is unknown.

Insertion of the masou salmon delta5-desaturase transgene into channel catfish had major effects on metabolism, physiology, body composition and growth of channel catfish. A major goal of increasing the percentage of n-3 fatty acids was achieved, but the objective of increasing total n-3 fatty acids was not. Selection and insertion of multiple desaturase transgenes may allow enhancement of both n-3 fatty acid percentage and total concentration, and should be evaluated. Potential adverse pleiotropic effects require further exploration. The compositional changes necessitate studies on texture, flavor and storage impacts for complete determination of the marketability and potential commercial application of desaturase transgenic channel catfish.

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Appendix

R code:

Depicting a histogram:

boxplot(Rdata$data~Rdata$ different group,ylab='variable',main='Dependent variable')

T-test to compare the differences between the two sets of data:

pairwise.t.test(Rdata$data,Rdata$different group, p.adj="none",pool.sd=F, var.eq=T)

F test to compare two variances:

transgenicmale = c(list of data separated by commas)

transgenicfemale = c (list of data separated by commas)

controlfemale= c(list of data separated by commas)

controlmale=c(list of data separated by commas)

var.test(group1, group2)