The Effects of the Masou Salmon Delta5-Desaturase Transgene on N-3 Fatty Acid Production in
F1 Transgenic Common Carp (*Cyprinus carpio*) and Channel Catfish (*Ictalurus punctatus*)

by

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Abstract

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 common carp, *Cyprinus carpio*, and channel catfish, *Ictalurus punctatus* as well as to determine pleiotropic effects of the transgene on growth, growth variation, and survival. Another important aspect of the experiment was to determine the validity of using smaller weights of muscle for lipid extraction than traditionally utilized. F1 generation common carp and channel catfish were spawned from confirmed transgenic parents. Muscle samples were obtained from these progeny and lipid extraction was performed. Quantification of n-3 fatty acid levels was accomplished through gas chromatography-mass spectrometry.

In general, n-3 fatty acid production was higher in transgenic individuals than in controls. Channel catfish showed no significant difference in n-3 fatty acid production because of low sample size, but trends towards increased production can be seen for n-3 fatty acids, in terms of total FAME percentage, α-linolenic acid (ALA) and docosahexaenoic acid (DHA), however these were not significantly different from controls ($P = 0.212$ and 0.207 respectively). The product on the n-6 side, arachidonic acid (AA), increased by 12.86% ($P = 0.8$). Precursors to n-6 delta5-desaturation, linoleic acid (LA) and dihomo-γ- linoleic acid (DGA) decreased 13.2% and 11.87% respectively ($P = 0.116$ and 0.8 respectively). On a mg/g weight basis for eicosapentaenoic acid (EPA) ($P = 0.089$), DHA ($P = 0.078$), total HUFA ($P = 0.056$) were higher in transgenic channel catfish. Fatty acid profiles for transgenic channel catfish were significantly more uniform compared to their control counter parts. Transgenic F1 common carp showed variation in lipid profile and higher level of saturated fatty acids when compared to controls.

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Transgenic F1 common carp showed higher variation in lipid profile and higher level of saturated fatty acids when compared to controls. Total fatty acid production of F1 desaturase common carp (9.55mg) and controls (9.93mg) per gram wet weight of muscle were not different (P = 0.68). As a percentage of FAME, desaturase F1 common carp showed a 1.14-fold decrease (P = 0.35) in n-3 fatty acid levels measured as a change in ALA, DHA, and EPA compared to controls. Individually, as a percentage of total fatty acids, ALA increased 20.72% (P = 0.18)

A large number of pleiotropic effects were attributed to desaturase gene insertion. The desaturase transgene appeared to have variable results on body weight in different families of channel catfish, and observed body weights of desaturase common carp were 20.4% lower than controls. This transgene made growth rates and fatty acid profiles more uniform. Observed disease resistance was improved in transgenic channel catfish, but tolerance of low dissolved oxygen was decreased.

The effects of the transgene varied between channel catfish and common carp. Desaturase transgenesis affected n-3 fatty acid production in a positive direction, showing promise for future work in this area, and when utilizing homozygous transgenic individuals in contrast to the heterozygous individuals utilized in the current study.

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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

PRSV – Papaya Ringspot Virus

PUFA - Polyunsaturated Fatty Acid

SD – Standard Deviation

1. INTRODUCTION:

Heart disease is currently the leading cause of death in America and has been since the early 1910s (Leading Causes of Death 1998;Hoyert, Ph, and Xu, 2012) . Since the early 1930's omega-3 fatty acids have been noted as essential for normal growth and human health, however overall awareness for their health benefits has increased dramatically in the last few decades (Health & Holman, 1998). At the turn of the 21st century nations like the United States and Canada began promoting omega-3 fatty acid nutritional supplementation stating that certain essential omega-3 fatty acids, mainly EPA and DHA, reduce the risk of coronary disease as well as helping to support the normal physical development of the brain, eyes, and nerves. Historically humans have consumed a 1:1 to 2:1 ratio of omega-6 to omega-3 fatty acids. Since the Industrial revolution, 150 years ago, there has been a shift towards an extreme consumption bias favoring omega-6 fatty acids in lieu of omega-3 fatty acids at a ratio of 15:1 -16.7:1 ratio (Simopoulos 2002).

Traditional sources of n-3 fatty acids include oils from many types of marine fish, however, these sources can be expensive. Contemporary research addresses utilizing available resources to produce these essential fatty acids and bring down their cost. Freshwater fish are an alternative to marine sources but they generally have lower n-3 fatty acid levels than their marine counterparts (Ugoala, Ndukwe, & Audu, 2008) . While salmon and tuna average 1825mg and 733mg, respectively, of EPA and DHA per 3oz serving, catfish only contain 151mg/serving. When accounting for all n-3 fatty acids, there are 2.15g per 100 grams of salmon in contrast to 0.6g per 100g tissue in carp and 0.28g per 100g tissue in catfish (Nettleton 2000; Gebauer, Psota, Harris, & Kris-etherton, 2006; Cheng et al. 2014). To nutritionally enrich freshwater fish like

catfish and carp to be more comparable with their marine counterparts for n-3 acids might enable greater marketing for producers and processors of these fish.

Omega-3 fatty acids, or n-3 acids, are polyunsaturated fatty acids with a double bond located between the third and fourth carbon atoms. The n-3 nomenclature is derived from the methyl grouping at one end of the molecule (Ruxton et al. 2004). Polyunsaturated fatty acids are long fatty acid chains that have more than 16 carbon atoms and have at least two or more double bonds. PUFAs are almost entirely manufactured by plants but many animals have the ability to convert from one form of PUFA to another by elongation and desaturation processes (Brett and Navarra 1997).

Among the PUFAs, α-linolenic acid (18:3 n-3, ALA), eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3; DHA) are all n-3 fatty acids necessary for human health (Simopoulos 2000). These acids are differentiated by the number of double bonds present in their structure, 3,5 and 6 for ALA, EPA, and DHA, respectively, all in a cis configuration. ALA can be metabolized to form EPA and EPA to DHA by desaturation processes (Ruxton et al. 2004). In contrast, Omega-6 fatty acids, are poly unsaturated fatty acids that have a double bond at the n-6 position. Omega-6 fatty acids important for human health include linolenic acid 18:2n-6 and arachidonic acid 20:4n-6. Metabolism of LA produces AA (Anderson and Ma 2009).

Omega-6 fatty acid metabolism of AA yields highly inflammatory products while omega-3 metabolites formed from EPA metabolism tend to be much less potent (Das 2006). These two metabolic processes are competitive because the same enzymes, cyclooxygenase and 5-lipoxygenase, are required for both pathways to operate. This pathway has a preference for ALA and n-3 metabolism but increased levels of LA in blood plasma shift enzyme metabolism towards the n-6 pathway (Ruxton et al. 2004) and metabolic competition by EPA results in a

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more n-3 dominated pathway. A healthy balance of n-3 to n-6 fatty acids in blood plasma is important as a heightened n-3 fatty ratio results in overall decreased production of inflammatory compounds, inhibited platelet aggregation, and vasoconstriction, all which are strongly correlated to cardiovascular disease (CVD) (Simopoulos 2002).

α-linolenic acid cannot be synthesized in humans as we lack the delta 12 and 15 desaturase enzymes necessary for production of this fatty acid. Dietary ALA and LA are commonly found in leafy vegetables and seeds. Once ingested ALA can be used directly or metabolized to form EPA and DHA but with low efficiency in humans (Anderson and Ma 2009). A comprehensive meta-analysis of many observational studies showed that individuals who were exposed to higher doses of ALA showed a lower risk of CVD (Pan et al. 2012)

Eicosapentaenoic acid 20:5n-3 is a PUFA n-3 fatty acid that humans can synthesize with low efficiency, but most commonly comes from dietary sources such as oily marine fish. These fish typically synthesize EPA from fatty acid precursors in their diet or obtain the fatty acid from marine algae that they consume. Increased levels of EPA in diets has been strongly correlated with diminished occurrence of CVD symptoms (Gebauer et al. 2006; Russo 2009).

DHA can be produced by the desaturation process but humans do not have the necessary enzymes for efficient metabolic production. In humans, small amounts of DHA can be obtained from EPA metabolism using DPA, docosapentaenoic acid (22:5), as an intermediate (Burdge 2002). DHA is an important acid for the development of the human brain and its continued function in adults. Deficiencies in DHA have been associated with many acute problems including fetal alcohol syndrome and more chronic effects like early onset of Alzheimer's disease (Horrocks and Yeo 1999).

N-3 Polyunsaturated fatty acids have many important roles in the human body. They function as integral membrane lipids in a variety of cells throughout the body and are necessary for the formation of eicosanoids (Dietary Reference Intakes 2002). The average intake of DHA and EPA is 0.1-0.2g per capita in North America and the average for ALA 1.4g. (Kris-etherton, Harris, Appel, and Committee 2002). Cardiac benefits have been seen with intakes of ALA at levels as low as 0.58g/day with greater benefits possible with levels as high as 2.81g/day. Intake of EPA+DHA is advised to be between 400-500mg/day (Gebauer et al. 2006).

When high levels of DHA and EPA are present they partially replace n-6 fatty acids in cell membranes such as arachidonic acid. This effect leads to decreased levels of prostaglandin E_2 metabolites, decreased thromboxane A_2 concentrations, and decreased B_4 formation, all of which can cause symptoms of CVD such as increased vasoconstriction and inflammation (Simopoulos 2000). Studies have suggested that increased omega-3 fatty acid intake could benefit many other chronic inflammatory related conditions including arthritis, cancer, diabetes and obesity (Wall et al. 2010). One of the reasons that n-3 fatty acids show cardiovascular benefits is their ability to decrease blood triglyceride levels where n-6 fatty acids do not (Lee and Lip 2003).

A 1997 study showed that individuals who were treated with fish oil (1.08g/day) for one year, after acute myocardial infarction, had significantly less chance of having a cardiac event or cardiac related mortality than placebo treatments (Singh 1997). Another larger study with 11,324 participants who had survived a significant cardiac event, myocardial infarction, were treated with a variety of methods. Those that were give n-3 PUFA's at a rate of 0.88g a day of EPA/DHA showed a significant reduction in future cardiac episodes (Gruppo and Commentary 1999).

In studies where direct fish consumption is monitored in lieu of fish oil, significant impact on cardiac symptoms can still be observed. A study that followed women at risk for coronary heart disease demonstrated that a significant trend was present between fish consumption as little as 1 meal a month increasing to 5 meals a week, and rate of coronary heart disease (Hu et al. 2003). Further studies have indicated that individuals who ate about one fish meal a week who were averaging about 665mg EPA/DHA a day had a 30-50% drop in chronic heart disease symptoms and related mortality when compared to individuals who rarely ate fish (Russo 2009).
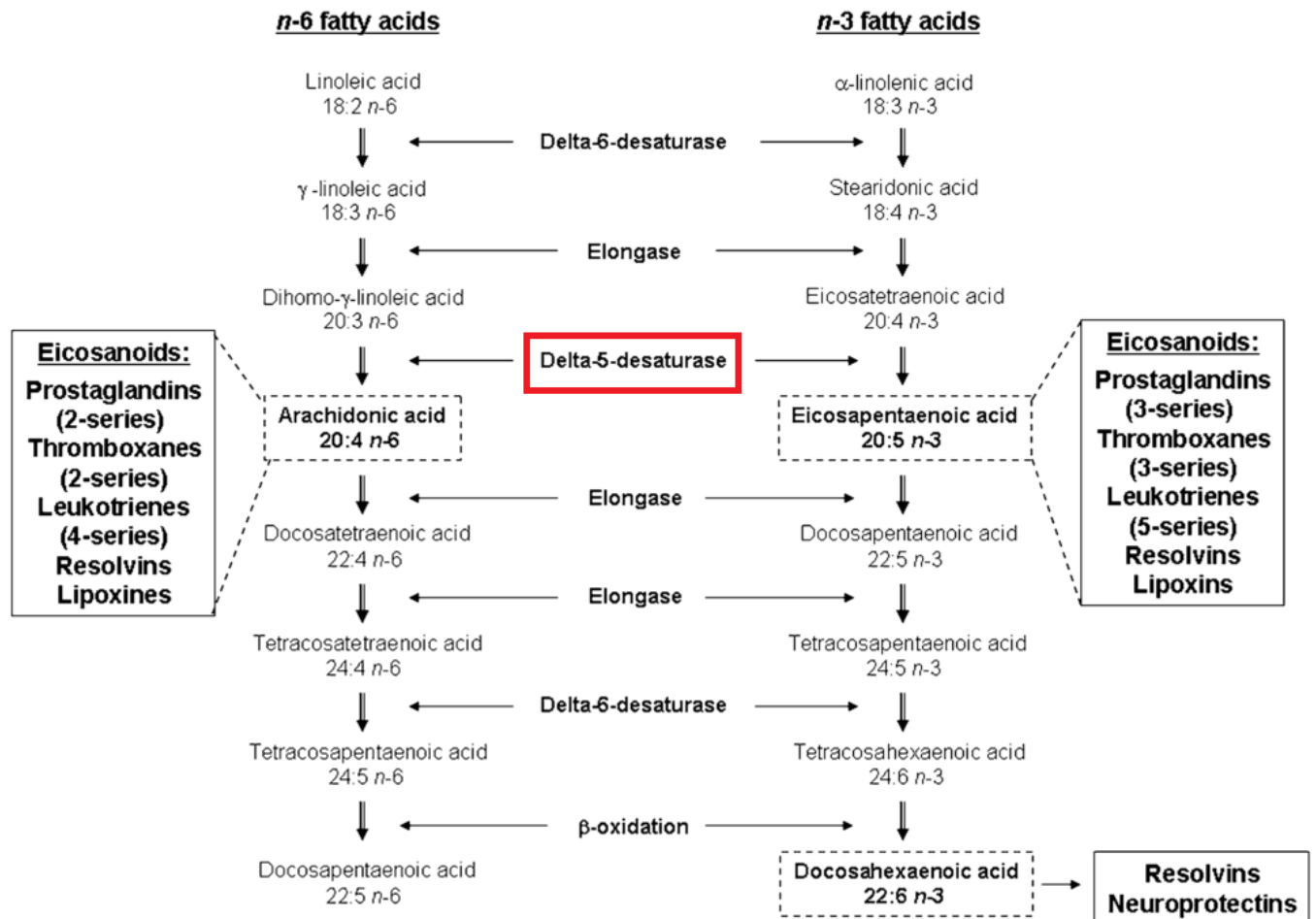
Production of n-3 and n-6 fatty acids relies on delta 5 and delta 6 desaturases as well as elongase function. A delta desaturase is an enzyme that functions to remove two hydrogen atoms at a certain point in a fatty acid chain, and replace them with a double bond. Delta 5 desaturase creates a double bond at the fifth position from the carboxyl end while delta 6 creates one at the 6th position. Delta 6 and 5 are membrane bound desaturases. Elongase is an enzyme that catalyzes 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. ALA can then be produced from linoleic acid through delta-15 desaturation (Lee et al. 2016). Vertebrates however, including common carp and channel catfish, do not have the ability to create essential PUFA's, 18:2 n-6 and 18:3 n-3, from these precursors, because they lack these necessary desaturases (Hastings et al. 2001). Instead these fish must get delta 5 and 6 desaturase precursors, 18:2 n-6 linoleic acid or 18:3 n-3 α-linolenic acid, from their diets.

In PUFA formation, fatty acids are first utilized by the delta 6 desaturase, elongase, delta 5 desaturase pathway (Figure 1) in the forms of linoleic acid 18:2 n-6 or α – linolenic acid 18:3

n-3. Delta 6 desaturase uses these substrates to produce either γ – linoleic acid 18:3 n-6 or stearidonic acid 18:4 n-3, respectively. Next elongase adds two carbon atoms onto the chains to form either dihomo-γ-linoleic acid (DGA) 20:3 n-6 or eicosatetraenoic acid (ETA) 20:4 n-3. Then these products can be utilized by delta-5-desaturase, forming arachidonic acid (AA) 20:4 n-6 or eicosapentaenoic acid (EPA) 20:5 n-3.

With the formation of these products eicosanoids are also produced. Production of AA leads to formation of 2-series prostaglandins, 2-series thromboxanes, and 4-series leukotrienes. In contrast production of EPA results in 3-series prostaglandins, 3-series thromboxanes, and 5-series leukotrienes (Wall et al. 2010), which are less inflammatory than their n-6 fatty acid metabolite counterparts (Simopoulos 2002).

Multiple elongase steps can then occur. If an n-6 fatty acid is being metabolized first docosatetraenoic acid 22:4 n-6 is produced from AA. If a second elongation occurs tetracosaetetaenoic acid 24:4 n-6 is formed. For n-3 fatty acids the first elongation forms docosapentaenoic acid (DPA) 22:5 n-3 with tetracosapentaenoic acid 24:5 n-3 formed with continued elongation. These secondary elongation productions can then be metabolized by delta-6-desaturase to form tetracosapentaenoic acid 24:5 n-6 or tetracosahexaenoic acid 24:6 n-3. B-oxidation, by peroxisomes, can then produce 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): linoleic acid (LA), arachidonic acid (AA), α-linoleic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

Genetic engineering has been long been utilized as a means of research as well as a method to increase natural productivity. The first patent for a genetically modified organism was issued in the US in 1980 for a bacterium that could be used to treat oil spills. In 1982 FDA approved the first medicine produced by a genetically modified organisms (GMO), insulin, which was produced by a genetically engineered strain of *E. coli*. In 1994 The Flavr Savr tomato, the first FDA approved GMO agriculture product, hit grocery store shelves, boasting a longer shelf life than its non-GMO counterparts. Genetic engineering has continued to improved agriculture crops in a variety of ways, increasing growth rate, resistance to disease, and resistance to climactic conditions.

Growth rate was successfully increased in cotton *Gossypium hirsutum* by integrating a phytochrome B transgene from *Arabidopsis thaliana*. The transgene successfully increased the photosynthetic rate of the plant by a multiple of more than four which led to a 46% increase in relative growth when compared to its non-transgenic counterpart (Rao et al. 2011).

Transgenic improvement of disease resistance has been shown in several agriculture products. Tobacco mosaic virus had long been a blight on tobacco production. To combat this virus, transgenic disease resistant tobacco was created by expressing a protein coat gene of the tobacco mosaic virus in the tobacco genome (Beachy 1999). This transgene was successful in produced viral resistance and accepted as a commercial agriculture product in the 1990s (James and Krattiger 1996). Increasing disease resistance through transgenesis has also been successful in the papaya. Papaya growth had been traditionally limited by papaya ringspot virus (PRSV) until the early 1990s. In 1988 PRSV- resistant transgenic papaya was produced by cloning the PRSV's protein coat gene and integrating it into the papaya genome. By the mid 1990's this

transgenic papaya had demonstrated its disease resistant qualities in test fields and had begun to allow papaya production to expand (Gonsalves 2004).

Climactic conditions can reduce growth and survival rates for many organisms. As temperatures increase relative to their historic norms, some species of plants have difficulty adjusting. Transgenes have been implored as a tool to solve this problem in the case of basmati rice, *Oryza sativa.* By introducing a transgenic heat shock protein, HSP101, from *Arabidopsis thaliana*, researchers were able to create a rice plant that showed increased tolerance to periods of high heat, and significantly increased growth rates in preceding recovery periods when compared to controls (Katiyar-agarwal, Agarwal, and Grover 2003).

Similar results can be seen when transgenic approaches are applied to animals, including fish. Dramatic growth rate increases have been shown in the case of Atlantic salmon, *Salmo salar*. Utilizing a chinook salmon growth hormone gene and an ocean pout promoter sequence, transgenic individuals had growth rates between 6 and 13 times greater than their non-transgenic counterparts in 8 month old smolt (Du et al. 1992; Fletcher et al. 1992). Three years later, transgenic salmon were reported to grow about 10 times the size of their control counterparts (Devlin et al. 1995; Cook et al. 2000b).

Transgenes have also been utilized to produce increased disease resistance across multiple fish species. A cecropin B transgene from a species of moth, *Hyalophora cecropia*, conveys increased disease resistance against pathogenic bacteria. In disease challenges utilizing the bacteria *Flavobacterium columnare* with channel catfish *Ictalurus punctatus*, transgenic individuals showed a 100% survival rate whereas controls only survived at a rate of 27.3%. In challenges with another common pathogen, *Edwardsiella ictaluri,* transgenic channel catfish survived at a rate of 40.7% compared to 14.8% in the control group (Dunham et al. 2002). The

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same transgene was tested in medaka, *Oryzias latipas,* challenging transgenic and control individuals with *Pseudomonas fluorescens* and *Vibrio anguillarum*. In these challenges control mortality was 40% while transgenic mortality was 10% (Sarmasik, Warr, and Chen 2002).

Salt water intrusion is expected to be a future problem accompanying climate change. To combat these effects there has been research into creating freshwater fish that are saline tolerant. This has been accomplished in the case of Nile tilapia, *Oreochromis niloticus*, utilizing transgenic techniques. DNA from sea bream, *Sparus aurata,* and artemia, *Artemia salina*, were used to create a line of Nile tilapia showing increased growth rate compared to controls in conditions as high as 32pt salinity (El-zaeem et al. 2011).

With the introduction of a transgene into the fish genome, unintended pleiotropic effects may result, either positive or negative. Improved feed conversion efficiency is one common pleiotropic effect when a growth hormone transgene is introduced. This has been demonstrated in common carp, channel catfish, Nile tilapia, and loach (Chatakondi 1995; Rahman et al. 2001; Dunham and Liu 2002; Nam et al. 2004).

Oxygen tolerance is another phenotypic trait prone to pleiotropic influence. Transgenic Nile tilapia, with the rtGH (Growth Hormone) transgene, demonstrated a longer average survival time at 0.4ppm dissolved oxygen than their non-transgenic full siblings (Chatakondi 1995; Dunham and Liu 2002). GH transgenic salmon showed higher oxygen demand after feeding compared to controls fed the same amount of feed (Lee et al. 2003)

Use of transgenes can also have a pleotropic effect on reproduction and viability of offspring. GH transgenic male medaka fish demonstrated a significant mating advantage over control counterparts engaging in 76% of all mating events in tanks with even numbers of

transgenic and control males (Howard, Dewoody, and Muir 2004). In contrast, transgenic Coho salmon show a decreased rate of successful mating (Bessey et al. 2004). Fry survival in the fastest growing transgenic salmon (30 times normal growth) is near zero. Progeny are subjected to elevated levels of endocrine stimulation resulting in early mortality (Devlin et al. 1995).

Changes in disease resistance have also been reported in transgenic fish. GH transgenic common carp show increased non-specific immune function with an increase of 18.7% in serum bactericidal activity (Wang 2006). Zebrafish with a delta 5 desaturase transgene have shown increased resistance to bacterial infection as a result of greater immunomodulatory activity (Wang, Tan, Jiao, You, and Zhang 2014). However not all transgenic fish have higher disease resistance. Transgenic salmon showed higher sensitivity to *Vibrio* than controls (Jhingan et al. 2003).

Morphological abnormalities have also been noted in transgenic fish. Fast growing transgenic salmon demonstrated morphological changes apparent in the head, fin, jaw, and operculum which were the result of increased cartilage and bone production (Devlin et al. 1995). Similar cranial deformities were documented in transgenic GH rainbow trout (Devlin et al. 2001). Transgenic pacific salmon developed gill filaments that were the same length as controls but lamellar spacing was decreased (Stevens and Devlin 2000). Transgenic Atlantic salmon, on the other hand, demonstrated similar lamellar spacing but gill filaments that were longer than controls (Stevens and Sutterlin 1999).

Pleiotropic effects can also make a GH transgenic more appealing to a consumer market. Both GH transgenic common carp and channel catfish showed increased protein and decreased levels of fat compared to their non-transgenic counterparts (Martinez et al. 1999) GH transgenic catfish also showed increases in number of mitochondria, glycogen globules, and muscle fibers,

as well as a decrease in fat globules in cells. As a result of these changes flavor and the texture of the transgenic catfish flesh was rated higher than that of controls (Dunham and Liu 2002).

Previous research has been conducted utilizing the delta 5 desaturase transgene. F1 and F2 zebrafish with this transgene showed increased levels of EPA and DHA, increasing 21% and 24% respectively (Alimuddin et al. 2007). Zebrafish with the transgene also showed increased disease resistance and immunomodulatory activity when challenged with *Vibrio vulnificus*. Transgenic fish showed a 350% increase in survival as well as a decrease in bacterial counts (Cheng et al. 2015). The delta 5 desaturase transgene has also been shown to increase n-3 fatty acid production in P-1 common carp. Common carp with the transgene had 7.3% higher n-3 fatty acid production than their control counterparts (Cheng et al. 2014).

The objectives of the current study were to evaluate the effectiveness of utilizing a delta 5 desaturase transgene from Masou salmon, *Oncorhynchus masou,* to increase omega-3 fatty acid production in F1 common carp and channel catfish, and to determine pleiotropic effects of the transgene on growth, growth variation, and survival. Non-lethal sampling is an important aspect of a genetic enhancement program and the amount of tissue required for fatty acid analysis would impact the size of the fish that can be sample and the survival of the fish post sampling. Thus, another objective was to determine the minimum quantity of muscle needed for fatty acid analysis.

2. MATERIALS AND METHODS:

2.1 IACUC Statement:

Channel catfish and common carp were utilized from the Fish Genetic Research Unit, School of fisheries, Aquaculture and Aquatic Sciences at Auburn University, Alabama. Research was conducted using procedures that were approved by the Institutional Animal Care and Use Committee (IACUC) of Auburn University.

2.2 Transgene Construction

The transgene (Figure 2) was built on 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: EU0981262.1). The D5D transgene was flanked by a beta-actin promoter from *Cyprinus carpio* (Accession ID: M241131.1) 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 at GenScript (USA Inc., Piscataway, NJ 08854, USA) (Cheng et al. 2014).
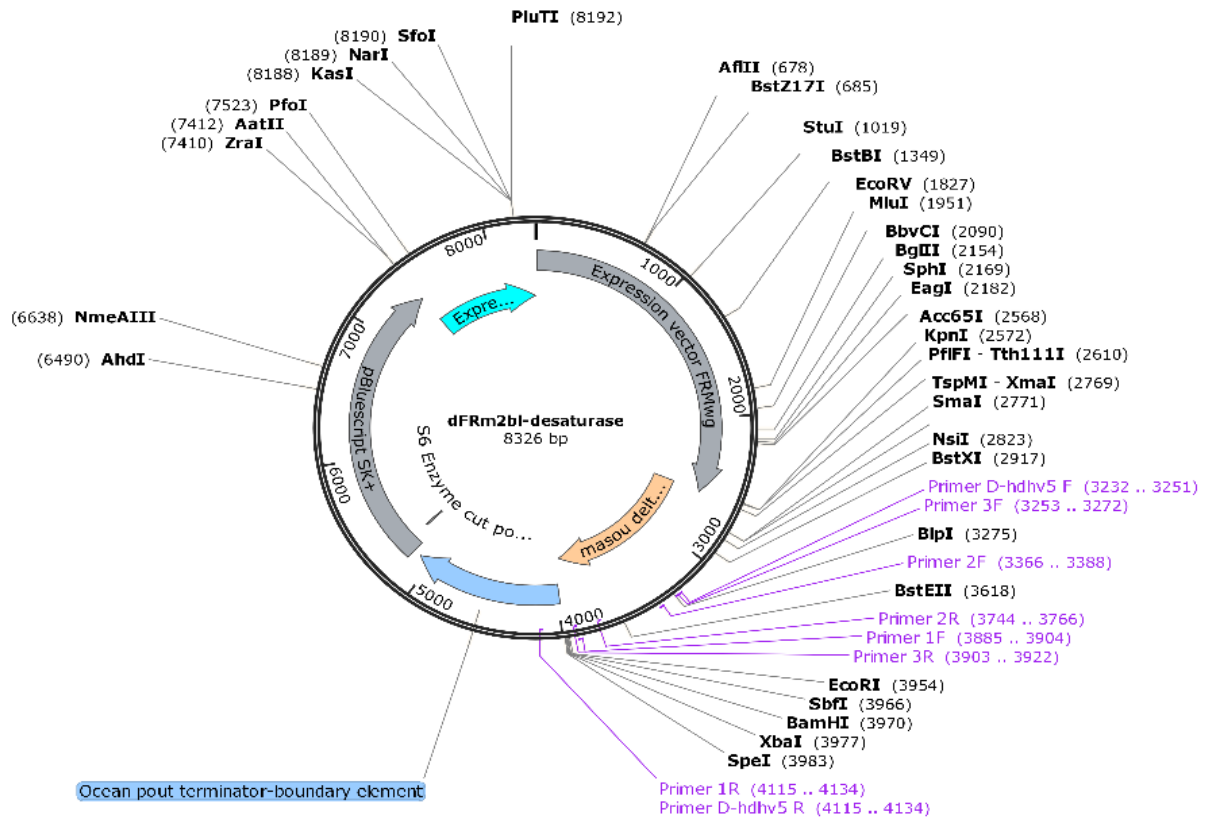
Figure 2: Restriction site map of the delta-5 desaturase transgene utilizing the *Oncorhynchus masou* delta-5 desaturase construct containing the beta actin promoter from *Cyprinus carpio.*

2.3 Broodstock Spawning

Sexually mature, desaturase transgenic, male and female common carp and channel catfish were harvested from the Fish Genetics Unit, EW Shell Research Center (Auburn University, AL). Fish were acclimated to 25-27°C.

Female common carp were injected with a 0.4mg/kg body weight (BW) priming dose of carp pituitary extract and then a resolving dose of 3.6mg/kg BW12 hours post injection to induce ovulation. Twelve hours post-resolving injection, fish were checked for eggs every 2 hours. If eggs were found the female was anesthetized in 100mg/L buffered MS-222 and hand stripped of eggs into greased spawning pans. Sperm was collected from male common carp by hand stripping of males. Sperm from transgenic males was used to fertilize eggs. Eggs were then placed in Holtfreter's solution 3.5g NaCl, 0.2g NaHCO3, 0.05g KCl, 333µl(300g in 500ml H2O) MgSO4, 333µl (150g in 500ml H2O) CaCl2, in 1.0L dechlorinated water with a pH of 7.0 to 7.5

Carp embryos began hatching in 3 days with a water temperature between 26-28°C. They consumed their yolk sac and began swim up stage 1 day post hatching. They were then fed artemia (Brine Shrimp Eggs, Carolina Biological) three times a day and stocked into a recirculating system with densities of 600 fish per 60L aquaria. After one month fry were fed Purina® AquaMax® Fry Powder (Purina, St. Louis, Mo) twice a day. As fry grew they were then fed Purina® AquaMax® Fry Starter 100. After further growth fry were moved to 60L aquaria at a stocking density of 50 fish per tank and fed Purina® AquaMax® Fry Starter 200 and 300 at least once a day. A total of 38 positive and negative individuals were pit tagged and placed into a communal growth tank and feeding was continued with AquaMax® Fry Starter 300 once daily. Desaturase common carp and controls of equal mix sex ratios were weighed at eighteen months of age.

Female channel catfish were intraperitoneally injected with 90ug/kg BW luteinizing hormone-releasing hormone analog (LHRHa) implant to induce ovulation. Fish were placed in labeled mesh bags in flow through holding tanks. Twenty four hours post injection, fish were checked for eggs every 4 hours. If eggs were found in the bag, the female was anesthetized with 100mg/L buffered MS-222 and hand stripped of eggs into greased spawning pans. Male desaturase transgenic channel catfish were euthanized. Testes were then collected from the fish, cleaned of blood and weighed. Testes were then crushed to release sperm. Eggs were fertilized with transgenic sperm and allowed to water harden for an hour before they were moved to mesh egg baskets suspended in paddlewheel troughs with adequate aeration.

Upon reaching swim up stage larvae were fed artemia or Purina® AquaMax® Fry Powder at early life stages two times a day. As fry grew larger they were fed AquaMax® Fry Starter 100. Fish were stocked at 500 fry per 110L flow through tank. At two months of age fish were restocked at a rate of 150 fry per 110L flow through tank. As they grew, fish were switched to AquaMax® Fry Starter 200 and then AquaMax® Fry Starter 300 containing 50% crude protein until they were large enough to take tissue samples to determine which fish were transgenic. After PCR was run, positive and negative individuals were pit tagged and stocked into 110L communal tanks at a density of 10 individuals per tank and feeding was continued with AquaMax® Fry Starter 300 once daily. Mortality data for both common carp and channel catfish were taken throughout the course of the experiment. After six months and twelve months of feeding mix sex desaturase channel catfish transgenic fry and controls fry were weighed. After eighteen months, male desaturase channel catfish transgenic fingerlings and control fingerlings were weighed.

2.4 Transgene Identification and Gene Quantification

Non-invasive sampling of tissue was taken from a single barbel clip that was used for DNA extraction. DNA extraction was accomplished utilizing proteinase K digestion followed by protein precipitation and ethanol precipitation utilizing the protocol described (Kurita, et al., 2004). DNA concentration was measured using UV-spectrophotometer (NanoDrop-2000, ThermoFisher Scientific Inc., Waltham, Ma.) and adjusted with the addition of sterilized water until concentration was about 500ng/µl. The DNA was run in a 1.2% agarose gel with ethidium bromide at 100 volts for 30 minutes to verify presence and quality of high molecular weight DNA.

PCR was performed on the sample to identify which samples contained the transgene. This was performed using a nested PCR design starting with the primers 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. Both rounds used the standard Platinum *Taq* (ThermoFisher Scientific Inc., Waltham, Ma) 10µl master mix with 2mM of each primer, 2mM of dNTPs, 15mM MgCl, and 10 unites of *Taq* polymerase. The first round utilized approximately 350ng of template DNA, while the second round utilized 1µL of the first round reaction as template. The amplification procedure for both rounds was as follows: initial denaturation for 5 min at 94 °C, followed by 40 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. The final reaction was run in a 1.2% agarose gel with ethidium bromide at 100 volts for 30 minutes to determine which samples amplified for the transgene. All PCRs were run with positive and negative control, the latter of which included both a non-transgenic channel catfish and a blank.

2.5 Lipid Extraction and Fatty Acid Analysis

Muscle samples from both channel catfish and common carp were prepared for lipid extraction by first freezing the sample with lipid nitrogen and then utilizing a pestle and mortar to grind the samples. Lipid extraction was accomplished following the protocol from Folch et al. (1957). Before extractions were conducted, 50ml tubes and corresponding flasks were washed and dried, using liquinox, along with their caps. Each flask and screw cap's weight was recorded. Sample tissues were transferred to mixing tubes via spatula. Weight of each sample was recorded. Samples ranged between 0.2 g and 2g depending on the amount of tissue available, and to test variability of results. Variability was measured as coefficient of variation between individual lipids, on a mg/g wet weight basis, in duplicate samples from the same fish. An average CV for each sample was calculated by taking the mean across all CV's for the individual fatty acids between duplicate samples. Common carp control samples were tested in duplicate at intervals of 0.0115g, 0.14g, 0.5g and 1.07g. Desaturase transgenic common carp samples were tested at 0.082g. Duplicates samples for desaturase common carp were not tested because tissue was not available. Channel catfish control samples were tested at 0.13g, 1.07g, and 2.05g. Desaturase transgenic channel catfish samples were tested at 2g. All channel catfish samples were tested in duplicate.

Chloroform-methanol (2:1) solution was then added to samples so that there was 17ml of solution per gram of tissue. Samples were then homogenized using a handheld tissue homogenizer for a minute per sample, or until tissue was sufficiently homogenized. The homogenizer was washed with 2:1 chloroform-methanol solution in between samples. A vacuum funnel and Erlenmeyer flask of appropriate size for the sample were chosen. Samples were poured into a stainless-steel vacuum filter and the water valve opened to apply suction with a

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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 remaining solution was poured into a flask and the solution volume was increased by adding 2:1 chloroform-methanol until the total volume was 20ml/g tissue sample. KCL solution (0.74% in water) was then added to each flask at 4ml/gram tissue sample. A stream of N_2 was then used to drive off air from the inside of the flask for 5-8 seconds. Flasks were then capped and shaken up and down 2 times. Samples were then left at room temperature to allow for phase separation overnight.

Once phase separation was accomplished, the upper phase of the solution was removed via kitasato flask. Flasks containing the samples were then placed in an N-EVAP containing 3.5L of 45^oC deionized water and N_2 streams opened to allow for the evaporation of the remaining liquid in the samples. Once dry, each flask was removed, the outside dried thoroughly and weighed. The initial weight of each flask was then subtracted from the post extraction weight to calculate the lipid weight of each sample. Then 50μl of internal standard (C 19:0, nonadecanoic acid methyl ester, Sigma- Aldrich, St. Louis, MO) was added to each sample (Internal standard was prepared in hexane at a concentration of 0.04g/5ml Hexanes). 1ml 0.5N KOH in MeOH was then added to each sample. Caps were then placed back onto the flasks and samples placed into a 70^oC water bath for 20 minutes. After 20 minutes, flasks were removed from the water bath and 1ml BF_3 in MeOH (14% BF_3, Sigma- Aldrich, St. Louis, MO) was added to flasks. Flasks were flushed with N_2 and returned to the 70^oC water bath for 40 minutes.

After 40 minutes, flasks were removed from the water bath and allowed to cool down to room temperature. Then 2 ml hexane and 2 ml saturated NaCl solution (25% NaCl in deionized water) were added to flasks. This solution was then vortexed for 15seconds. Then 9 inch

disposable Pasteur pipettes were used to siphon the upper layer and transfer it to test tubes with glass fiber filters and ammonia sulfate. The remaining hexane was then evaporated with N_2 and a calculated amount of hexane was added to samples to reach a concentration of 10 mg lipid/ml of hexane. Samples were mixed and then pipetted to injection vials which were labeled and stored at -20^oC until they could be injected into the gas chromatograph (GC). Do to equipment problems some samples had to be stored for two months before they could be retested for fatty acid content because of issues with gas chromatography equipment. During this time samples were kept in a -20^oC freezer in 4ml borosilicate glass vials with PTFE caps tightly secured. This was done with 0.2g, 0.5g, and 1g control carp samples as well as 0.2g and 1g catfish samples.

2.6 Gas Chromatography

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 done with 5875 mass spectrometer. Peaks were identified 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^oC for 2 minutes, then increased to 183C at a rate of 50^oC per minute. The temperature was then increased to 230^oC at a rate of 3^oC per minute, where it was held for 1 minute. A 2μL sample was run for each sample using splitless mode and FA levels were expressed as a percentage of total identified FAMES and on a per gram of wet tissue basis.

2.7 Statistical Analysis

Mortality data was collected along with fish samples for PCR analysis. Statistical analysis was done using R version 3.3.1 (code outline available in appendix). Test of equal or given proportions ($P < 0.05$) was used to determine significance of mortality. P values for

differences in body weight and lipid concentrations were calculated using a two-sample t-test ($P < 0.05$) and analysis of weight variation was conducted utilizing an F test to compare two variances ($P < 0.05$). Significance of weight of sample on lipid variability was calculated using linear regression analysis ($P < 0.05$).

3. RESULTS

3.1 N-3 Fatty Acid Production in Transgenic Common Carp

Total fatty acid production of F1 desaturase common carp (9.55mg (n=5)) and controls (9.93mg (n=2)) per gram wet weight of muscle were not different ($P = 0.68$). An F-Test was conducted to compare variances between fish in total fatty acid production and found that desaturase fish were not different ($P = 0.51$) from their control counterparts.

As a percentage of FAME, desaturase F1 common carp showed a 1.14-fold decrease ($P = 0.35$) in n-3 fatty acid levels measured as a change in ALA, DHA, and EPA compared to control counterparts. Individually, as a percentage of total fatty acids, ALA increased 20.72% ($P = 0.18$). Saturated fatty acids were observed to be higher, 3.66mg, in desaturase common carp in terms of mg/g wet weight, than in controls, 2.93mg ($P = 0.25$).

EPA, the direct results of n-3 delta 5 desaturation, was 3.15% lower as a percentage of total FAME ($P = 0.94$). The product on the n-6 side, AA, decreased 2.44% ($P = 0.9$). The precursor to delta 5 desaturation on the n-6 side of the pathway, DGA, was 30.24% higher in transgenic individuals but not significantly ($P = 0.14$). Monounsaturated fatty acid C22:1, eurcic acid, was decreased by 56.3% in desaturase individuals, as a percentage of total fatty acids, but not significantly ($P = 0.23$). Effects of the delta 5 desaturase transgene on the fatty acid profile of desaturase common carp compared to controls are illustrated in Figure 3. Significant results were

found on mg per gram weight basis in the case of C 18:1n9 (P = 0.015), total monounsaturated fatty acids (P = 0.015), and total n-6 fatty acids (P = 0.03).

Average coefficient of variation was calculated across all fatty acids, on a mg/g wet weight basis, between control and desaturase common carp in terms of variation between individual fish. Desaturase carp had an average variation in fatty acid levels of 23.34% while CV across controls was 21.74%. Significance between the two variances in fatty acid profiles was calculated and the result was not significant (P = 0.71).

	C 11:0	C 14:0	C 15:0	C 16:0	C 16:1	C 17:0	C 18:0	C 18:1n9	C 18:2n6 LA	C 18:3n3 ALA	C 20:0	C 20:1	C 20:2n-6	C 20:3n-6 DGA	C 20:4n-6 AA	C 20:5n-3 EPA	C 22:1	C 22:2n-6	C 22:6n-3 DHA
percent change	12.4704602	0.89534075	22.747536	28.8603188	-31.209521	43.3204737	50.7043091	-16.118794	-25.163832	20.7129064	57.9139344	-5.1049386	35.4205861	30.2428043	-2.4370399	-3.1592771	-56.358357	30.105596	-26.473485

Figure 3: Percentage change in fatty acids in muscle of masou salmon delta 5- desaturase transgenic common carp, *Cyprinus carpio*, (n = 5) compared to control common carp (n=2) grown in recirculating tanks.

Table 1: Mean mg/g weight faty acids and percent fatty acid methyl ester (FAME) for F1 Common carp, *Cyprinus carpio,* transgenic for delta-5 desaturase compared to control common carp from the current study, and control common carp from (Cheng et al. 2014)[a]

mg/g Wet Weight	Cheng 2014 Controls	Control Common Carp	Transgneic Common Carp
Total Lipid %	3.32	0.99	0.95
16:0	11.42	1.82	2.50
18:0	2.69	0.86	1.20
18:1n-9	11.92	1.72	1.34**
18:2n-6	4.57	0.74	0.52
18:3n-3	0.52	0.16	0.18
20:4n-6	0.06	0.68	0.61
20:5n-3	3.37	0.72	0.65
22:6n-3	5.15	2.10	1.43
Saturates		2.93	3.66
Monounsaturates		2.20	1.71**
PUFA		3.26	2.85
HUFA	8.07	3.49	2.69
Total n-3	8.60	2.97	2.25
Total n-6		1.53	1.26**
FAME %			
16:0	18.54	18.35	21.55
18:0	4.36	8.61	12.49
18:1n-9	19.36	17.35	14.97
18:2n-6	7.42	7.52	5.89
18:3n-3	0.83	1.58	2.01
20:4n-6	0.10	6.79	6.43
20:5n-3	5.46	7.23	6.95
22:6n-3	8.35	21.07	16.23
Saturates		29.55	37.21
Monounsaturates		22.12	19.10
PUFA		11.80	11.64
HUFA	13.60	35.06	30.47
Total n-3	11.42	29.85	25.53
Total n-6		15.40	14.35

[a] Totals calculated as follows:

Saturates: 14:0, 15:0, 16:0, 18:0, 22:0, Internal standard 19:0 not included.

Monounsaturates: 16:1, 18:1, 20:1

PUFA: 18:2, 18:3, 20:2, 20:3, 22:2

HUFA: 20:4, 20:5, 22:6

Total n-3: 18:3n-3, 20:5n-3, 22:6n-3

Total n-6: 18:2n-6, 20:3n-6, 20:4n-6

* Significant at $P < 0.1$

**Significant at $P < 0.05$

3.2 N-3 Fatty Acid Production in Transgenic Channel Catfish

Total fatty acid production of F1 desaturase channel catfish (18.23mg (n=2)) and controls (23.8 mg (n=3)) were not different (P = 0.22). The CV for total fatty acid production in desaturase fish was 0.71% while controls had a variation 18.77%. Variances were compared between fish in total fatty acid production and found that desaturase fish had lower variation than controls(P = 0.012).

As a percentage of fatty acids, desaturase F1 channel catfish showed a 1.11-fold increase in n-3 fatty acid levels measured as a change in ALA, DHA, and EPA compared to control counterparts (P = 0.54). Levels of n-6 fatty acids, LA, AA, and DGA, decreased 1.11-fold in transgenic individuals (P = 0.2). Individually n-3 fatty acids ALA and DHA increased by 44.34% and 13.2% respectively, however, these were not significantly different from controls (P = 0.21 and 0.21 respectively). EPA, the direct results of n-3 delta 5 desaturation, was 0.94% lower as a percentage of total FAME (P = 0.81). The product on the n-6 side, AA, increased by 12.86% (P = 0.8). Precursors to n-6 delta5-desaturation, LA and DGA, decreased 13.2% and 11.87% respectively (P = 0.116 and 0.8 respectively). Significant results at P < 0.1 were seen on a mg/g weight basis for EPA (P = 0.089), DHA (P = 0.078), total HUFA (P = 0.056). Total n-3 fatty acids decreased (P = 0.043). Effects of the delta 5 desaturase transgene on the fatty acid profile of desaturase channel catfish compared to controls are illustrated in Figure 4.

Average coefficient of variation, as a on a mg/g wet weight basis, was calculated across all fatty acids between control and desaturase fish in terms of variation between individual fish. Desaturase fish had an average variation in fatty acid levels of 7.11% while controls showed increased variation with a CV of 22.07%. Significance between the two variances in fatty acid

profiles was calculated and the variance of the fatty acids of the controls was higher than that of the transgenic individuals (P = 0.0007).
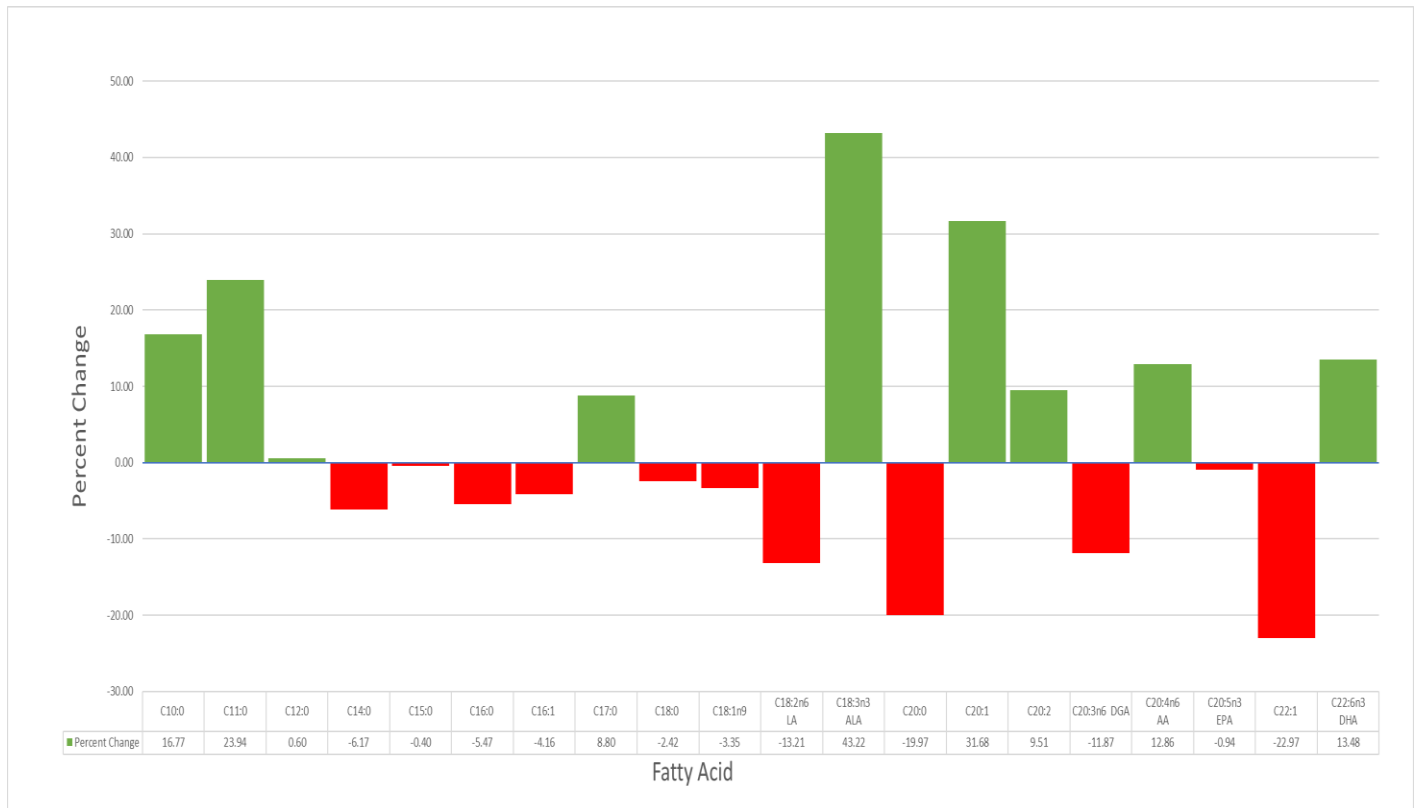
Figure 4: Percentage change in fatty acids in muscle of masou salmon delta 5- deaturase transgenic channel catfish, (n = 2, two replicates per fish), *Ictalurus punctatus,* grown in flow through tanks relative to controls (n=3). No significant changes in fatty acid level were observed α = 0.05.

Table 2: Mean mg/g weight fatty acids and percent fatty acid methyl ester (FAME) for F1 Channel catfish, *Ictalurus punctatus,* transgenic for delta-5 desaturase compared to control channel catfish[b]

mg/g Wet Weight	Control Channel Catfish	Transgenic Channel Catfish
Total Lipid %	2.38	1.82
16:0	2.69	1.95
18:0	0.74	0.55
18:1n-9	5.72	4.24
18:2n-6	3.43	2.28
18:3n-3	0.17	0.18
20:4n-6	0.32	0.28
20:5n-3	1.97	1.49*
22:6n-3	6.13	5.33*
Saturates	4.27	3.08
Monounsaturates	6.84	5.14
PUFA	3.84	2.64
HUFA	8.43	7.11*
Total n-3	8.27	7.01**
Total n-6	3.91	2.66
FAME %		
16:0	11.29	10.67
18:0	3.11	3.04
18:1n-9	24.03	23.23
18:2n-6	14.42	12.52
18:3n-3	0.70	1.00
20:4n-6	1.35	1.53
20:5n-3	8.28	8.20
22:6n-3	25.77	29.25
Saturates	17.94	16.88
Monounsaturates	28.97	27.66
PUFA	49.17	50.97
HUFA	35.40	38.97
Total n-3	34.75	38.45
Total n-6	16.41	14.60

[b]Totals calculated as follows:

Saturates: 14:0, 15:0, 16:0, 18:0, 20:0 Internal standard 19:0 not included.

Monounsaturates: 16:1, 18:1, 20:1

PUFA: 18:2, 18:3, 20:2, 20:3

HUFA: 20:4, 20:5, 22:6

Total n-3: 18:3n-3, 20:5n-3, 22:6n-3

Total n-6: 18:2n-6, 20:3n-6, 20:4n-6

* Significant at $P < 0.1$

**Significant at $P < 0.05$

3.3 Pleiotropic Effects

One month 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. The population of this tank was 28.2% transgenic. The observed percentage of transgenic fish among the mortalities, 20.8% was lower than the percentage of transgenic fish in the initial population, 28.2%, but these two percentages were not significantly different.

The average body weight of 15 desaturase transgenic fry was 10.1g with a standard deviation (SD) of 2.2g and a coefficient of variation (CV) of 21.9%. The average body weight of 35 control fish was 11.8g with a SD of 4.5g and a CV of 38.7%. Control channel catfish were 16.8% larger than F1 desaturase channel catfish (P = 0.05). F1 desaturase channel catfish had less body weight variation compared to controls (P = 0.0026).

The mean body weight of 12-month-old mix sex transgenic desaturase catfish (n = 3) was 113g with a SD of 20g and a CV of 17.65%. Full sibling controls (n= 30) had a mean body weight of 76g with a SD of 32g and a CV of 42.7%. Desaturase transgenic channel catfish were observed to be 48.6% larger than their controls for body weight (P = 0.076). Variance in body weight was not statically significant (P = 0.42).

The mean weight of 18-month-old transgenic desaturase channel catfish males (n = 2) was 431.5g with a SD of 72.5g and a CV of 16.8%. Male controls (n = 5) had a mean weight of 315.8g with a SD of 92.88g and a CV of 29.4%. Male desaturase catfish were observed to be 37% larger than their controls for body weight, however, the difference was not significant (P = 0.16). Variance in body weight was also not different between the two genetic groups (P = 0.38).

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 of this tank was 16.1% transgenic. The observed percentage of transgenic fish among

the mortalities, 42.9% was greater than the percentage of transgenic fish in the initial population,

16.1% (P = 0.055).

The mean body weight of transgenic desaturase common carp was 37.1g with a

SD of 13.7g and a CV of 36.9%. The mean body weight of non-transgenic common carp was

46.6g with a SD of 39g and a CV of 83.7%. The observed apparent difference in body weight for

the two groups was not different (P = 0.23). Variance in body weight between the transgenic and

non-transgenic individuals was different (P = 0.002).

3.4 Lipid Extraction Variability

Channel catfish and common carp control samples were extracted for lipids followed by

transesterification, using varying amounts of tissue. Samples from common carp controls

weighing 0.115g, 0.14g, 0.5g and 1.07g had a coefficient of variation of 22.19, 17.17, 18.21, and

18.95%, respectively for individual lipids on a mg/g wet weight basis. CV among the five

transgenic desaturase common carp samples (0.082g per sample) from individual fish, was

calculated at 22.14%.

In the case of channel catfish controls, the 0.13g, 1.07 and 2.05g samples had CVs of

31.06, 13.31 and 14.96% respectively, between individual lipids on a mg/g wet weight basis.

Two gram samples of desaturase channel catfish had an average CV of 9.48%. CV between 2g

control channel catfish and 2g desaturase channel catfish were not different (P = 0.26). The

regression equation for the effect of sample size on CV (across samples from both species)

between individual lipids, on a mg/g wet weight basis, between duplicate samples from the same

fish was: $Y = -4.4743x + 22.789$ with an R^2 of 0.4011 (Figure 5). The influence of sample weight on size variability was not significant ($P = 0.151$).

Average mg/g fatty acid varied across sample sizes. Samples from common carp controls weighing 0.115g, 0.14g, 0.5g and 1.07g had fatty acid levels of 9.9, 6.25, 2.94, and 3.09 mg/g wet weight. In the case of channel catfish controls, the 0.13g, 1.07 and 2.05g samples had fatty acids levels of 10.91, 6.89, and 23.8 mg/g wet weight respectively.
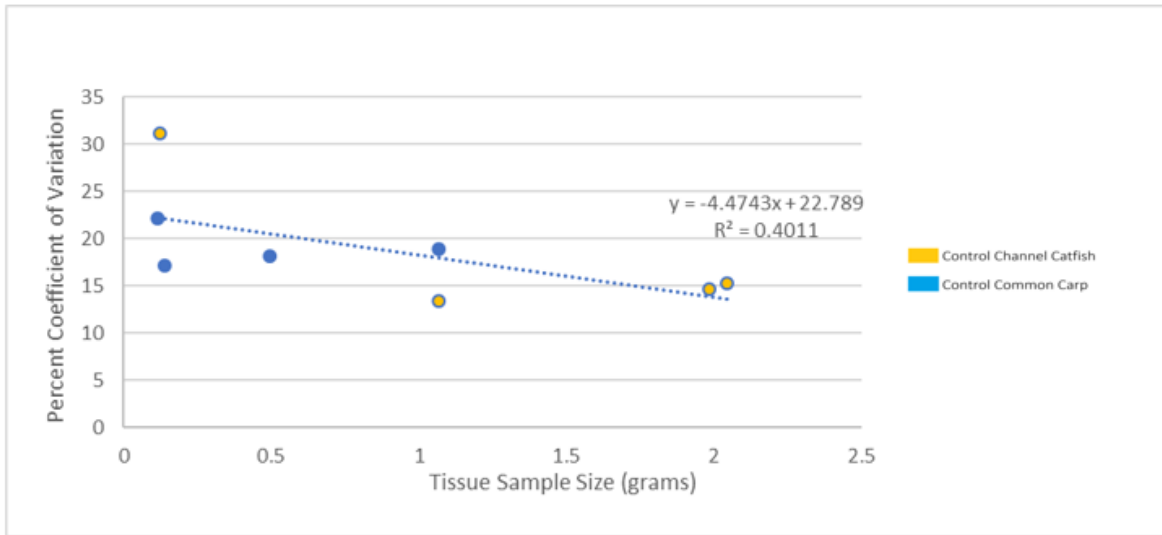
Figure 5: Percent CV as measured on a mg/g wet weight basis, across duplicate samples for different tissue sample weights in common carp*, Cyprinus carpio,* and channel catfish, *Ictalurus punctatus*. Tissue sample weight did not have a significant effect on lipid profile variation (P = 0.15). The coefficient of correlation for the regression is -0.633.

3.5 Long Term Storage Effects on N-3 Fatty Acid Samples

Samples retested after two months had largely different levels of n-3 fatty acids (carp 2.64mg to 0.44mg P = 0.009, catfish 8.27mg to 0.51mg P = 0.0018). In terms of mg/g wet weight, across sample sizes, carp samples decreased in amount of DHA (1.9mg to 0.355mg, P = 0.002), EPA (0.69mg to 0.068mg, P = 0.07), and ALA (0.047mg to 0.013mg, P = 0.009), while levels of palmitic acid increased (0.36mg to 1.436mg, P = 0.12). Total fatty acid levels decreased (6.425mg to 3.99mg, P = 0.061) (Figure 6). In channel catfish results were similar with significant decreases in DHA (6.13mg to 0.27mg, P = 0.0007), EPA (1.97mg to 0.183mg, P =0.008) and ALA (0.166mg to 0.056mg, P = 0.14). Palmitic acid levels unchanged on a mg per gram basis (2.68mg to 2.69mg, P = 0.825) but were altered as a percent of total fatty acids (11.89% to 35.35%, P = 0.011). Total fatty acids for the samples were different (23.8mg to 8.37mg, P = 0.02) (Figure 7).
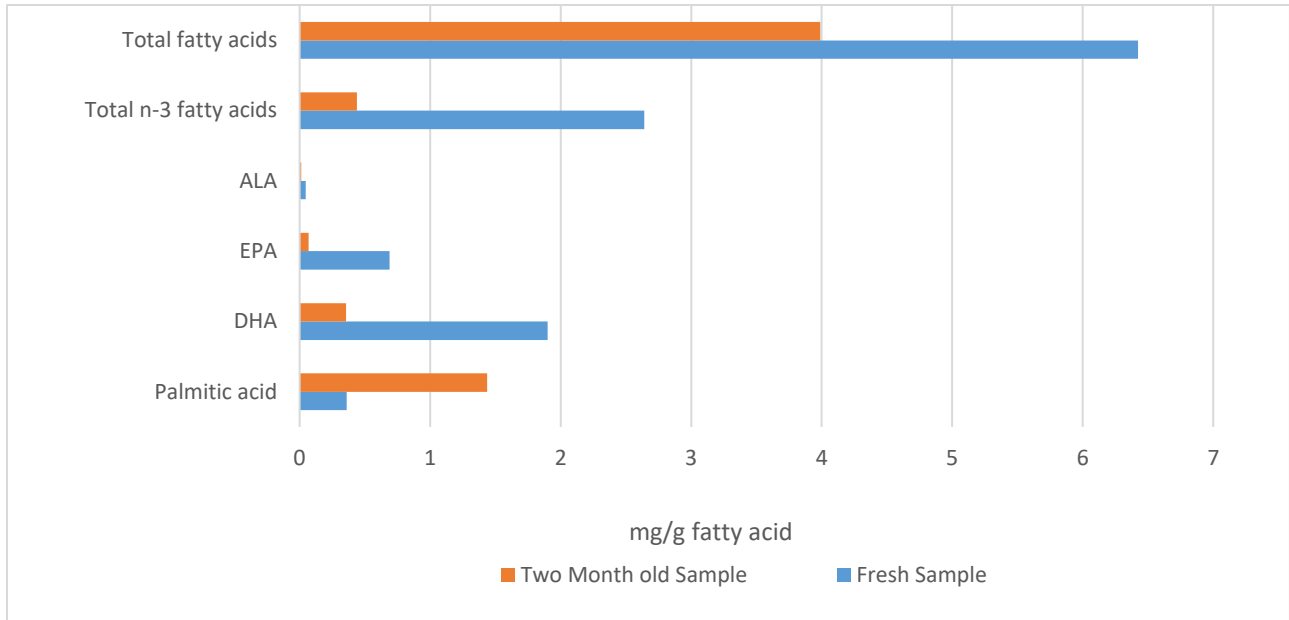
Figure 6: Effects of long term freezer storage on n-3 fatty acid and total fatty acid composition of transesterified lipid extraction samples from common carp (*Cyprinus carpio)* muscle tissue. All values are statistically significant ($P < 0.05$).
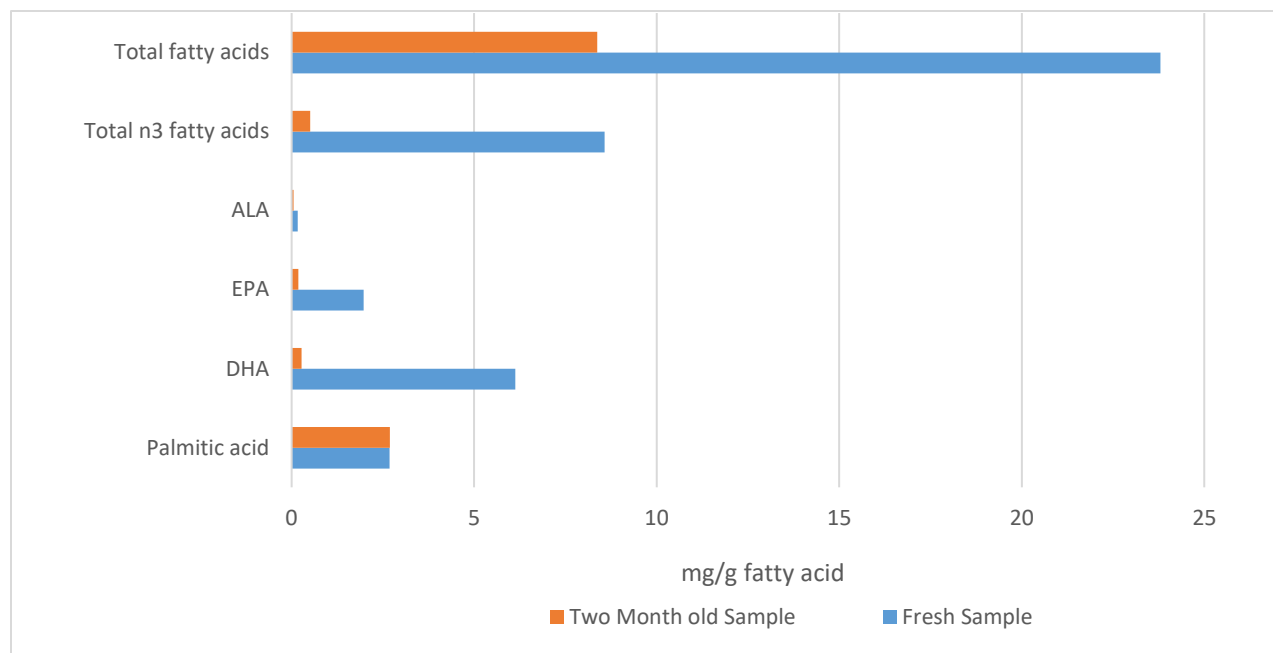
Figure 7: Effects of long term freezer storage on n-3 fatty acid and total fatty acid composition of transesterified lipid extraction samples from channel catfish (*Ictalurus punctatus*) muscle tissue. Values for total fatty acids, total n-3 fatty acids, ALA, EPA, and DHA are statistically significant (P < 0.05).

4. DISCUSSION:

In the present study, a masou salmon delta-5 desaturation transgene was tested to determine its effect on n-3 fatty acid production in common carp (*Cyprinus carpio*) and channel catfish (*Ictalurus punctatus*). Lipid extraction and transesterification was used to determine the levels of fatty acid present in control and transgenic tissue samples ranging from 0.08g to 2.05g. Pleiotropic effects of the transgene on growth, growth variation, and survival were assessed.

In common carp (*Cyprinus carpio*) there were large changes to fatty acid levels and lipid profile between control and transgenic individuals. In common carp, there was an overall decrease in n-3 and n-6 fatty acids in transgenic individuals for both the mg/g wet weight basis and as a percentage of total FAME. Overall fatty acid production remained unchanged between the two groups but lower than results from Cheng et al. (2014) experiment. In transgenic individuals, there observed levels of saturated fatty acids were higher, but the differences were not significant. If n-3 fatty acid production is compared to fatty acid levels reported for the controls of Cheng et al. (2014) there was a 106% increase seen in total n-3 fatty acid production in transgenic individuals as a percentage of total FAME. Reported levels of n-3 fatty acids in other studies show common carp muscle tissue containing 2.42mg/g, (Kminkova et. al. 2001), which is consistent with the results in the current study, 2.25mg/g for transgenic individuals and 2.97mg/g for control fish.

In channel catfish (*Ictalurus punctatus*) transgenic individuals produced lower levels of total fatty acids than their control counterparts. On a mg/g weight basis all fatty acids reported were lower in desaturase individuals compared to their control counterparts. However, as a percentage of total FAME's n-3 fatty acids are increased in transgenic individuals compared to their controls. The delta 5 desaturase products on the n-6 side of the pathway were also increased

in transgenic individuals while their precursors were in lower concentrations. While overall fatty acid levels might be lower in transgenic individuals, the fatty acids that are present in transgenic individuals are richer in n-3 fatty acids than in controls. Desaturase transgenic channel catfish also showed significantly lower lipid variability than their control counterparts. Desaturase transgenic common carp lipid variability was not different from controls.

While lipid results did not mirror previous work done in other species (Alimuddin et al. 2007; Cheng et al. 2014), the delta 5 desaturase transgene did successfully increase n-3 fatty acid levels in F1 channel catfish and showed alteration in fatty acid profiles in F1 common carp. Transgenes do not always produce the same effect in different species and can be strongly influenced by their position and surrounding elements in the genome. F1 Nile tilapia transgenic for a sockeye GH transgene showed no increase in growth rate, while transgenic salmon possessing the same transgene showed large enhancements in growth rate (Rahman et al. 1998). Transgenes can be silenced or have altered production by natural processes and epigenetic effects (Matzke and Matzke 1998). A combination of these effects are possible in the case of altered n-3 fatty acid production in F1 common carp.

Growth rate alterations were apparent across study groups, but not consistent. In channel catfish, increased growth rates were observed in both twelve-month-old and eighteen-month-old fish from the same genetic line while another family showed decreased growth rates at six months of age. Common carp, while not statistically different, did have lower average body weights at eighteen months of age. Varying growth rate results could be caused by age effects, strain effects, or the impact of differing insertion sites. More research needs to be done to determine the full effect of the transgene on growth rate.

Decreased size variation was observed across species as well as age classes. Mixed sex common carp and channel catfish fry showed significantly less size variation than their non-transgenic, full-sibling control. Male channel catfish fingerlings, while not significantly different, exhibited the observed trend of decreased size variation. One of the major problems that commercial catfish farmers face is size variability when sending their fish to processors. Decreasing variability in size is important as it produces a more uniform fish for processors to handle and help prevent price decreases given to farmers when over- or under-sized catfish are harvested.

Early mortality was not significantly different between control and desaturase channel catfish. However, the observed trend was consistent with other research indicating possible increased disease resistance, immunomodulatory activity, and survival in delta 5 desaturase transgenic fish (Wang et al. 2014; Cheng et al. 2015). Increasing resistance to a variety of pathogens is an important goal of transgenic research. Finding a beneficial transgene that also includes a pleiotropic effect of increasing diseased resistance would increase the value and potential acceptance of transgenic fish commercially.

Increased mortality of F1 desaturase channel catfish fingerlings, caused by hypoxic conditions, was approaching significance in our study. This may be because desaturase fingerlings had larger rates of oxygen consumption than their control counterparts based on their increased size or had increased metabolic rates (Cook et al. 2000a).

Variability from lipid extraction decreased as the weight of individual samples increased, but not significantly. Working with larger samples improved accuracy and transfer efficiency when conducting procedure steps that may introduce error. The regression for lipid profile variability between replicates against weight of samples showed a strong negative linear

relationship between data points. Depending on level of preciseness required and availability of tissue, an appropriate sample size can be approximated based upon the regression equation. To keep variation below 20% muscle samples should be 0.6g in size or greater but samples as small as 0.08g can be effectively utilized. Channel catfish and common carp variability across fatty acids were similar (19.77% compared to 18.94%) showing that there should not be a species effect on fatty acid variability.

In a genetics enhancement program, it is important to be able conduct non-lethal sampling. By minimizing the amount of tissue required to conduct fatty acid analysis, smaller fish can be sampled, and increased survival rates obtained post sampling. These results demonstrate that depending on level of accuracy required, muscle samples as small as 0.08g can be effectively used in lipid extraction. Even smaller samples may be utilized effectively but further testing is needed to determine the minimum necessary.

Increased storage time under -20°C conditions led to a significant decrease in total fatty acids and n-3 fatty acids in control samples when compared to fresh samples. To ensure that GC-MS results are accurate, lipid samples should be run soon after lipid extraction and transesterification are completed. Our experience indicates that delaying GC-MS analysis for a period of two months greatly diminishes result viability.

In future studies, use of non-lethal barbel and adipose clip samples should be evaluated for lipid extraction as they can be readily taken with minimal impact to fish. We conducted preliminary trails, and procedures to use these potentially useful tissues. Procedures will need to be altered as the structure of these tissues made lipid extraction difficult. Also, strain effects on desaturase transgene alteration of fatty acid profiles should be evaluated as strain performance of transgenic individuals (Devlin 2001). In the current study, transgenic individuals were all

heterozygous, and homozygous individuals might be expected to have greater n-3 fatty acid

production in the next generation. Finally, in the current study, double electroporation was

utilized as the method of transgene integration. Utilizing a more targeted gene insertion approach

like CRISPER/Cas9 may show better results and allow for further understanding of the function

of the transgene.

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Appendix – R Code Utilized

Test of equal or given proportions:

Transgenicmortality = c(3/5)

Controlmortality = c(4/26)

Prop.test(x = c(3,4), n = c(5,26), alternative = "greater")

Two-sample t-test:

Transgenicweight = c(list of weights separated by commas)

Controlweight = c(list of weights separated by commas)

t.test(transgenicweight, controlweight)

F test to compare two variances:

Transgenicweight = c(list of weights separated by commas)

Controlweight = c(list of weights separated by commas)

var.test(transgenicweight, controlweight)

Linear regression analysis:

Sampleweight = data.frame(

Coefficientofvariation = c(coefficient of variations separated by commas)

Sampleweight = c(sample weights separated by commas)

)

Regression = lm(coefficientofvariation ~ sampleweight, data = sampleweight)

Summary(regression)