Genetic Technologies for Growth Enhancement in Catfish

by

Michael Philip Coogan

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Approved by

Rex Dunham, Chair, Professor of Fisheries, Aquaculture and Aquatic Sciences
Ian Butts, Assistant Professor of Fisheries, Aquaculture and Aquatic Sciences
Charles Chen, Professor of Crop, Soil and Environmental Sciences
Joana Diller, Associate Professor of Biological Sciences

Abstract

The effects of CRISPR/Cas9 knockout of the myostatin (MSTN) gene in channel catfish, *Ictalurus punctatus,* was investigated. A total of 209 fish survived microinjection over 3 years with an average mutation rate of 58%. Successful generation of MSTN F1 mutants was achieved in 2019 by individually mating two pairs of control females with MSTN mutant males. The offspring of both families inherited the mutation at a high rate of 88%. Growth was generally higher in MSTN mutants when compared to controls at all life stages and in both pond and tank environments. Heterozygous F1 mutants were 218% larger than controls at the stocker stage in ponds. Mean expression level of MSTN was 2.90 times higher in controls than in MSTN F1 progeny ($p=0.009$). When challenged with *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC), MSTN mutants performed equally or better than controls.

The effects of CRISPR/Cas9 knockout of the melanocortin-4 recepter (MC4R) gene in channel catfish was investigated. Growth was generally higher in MC4R mutants when compared to controls at all life stages and in pond and tank environments. There were no significant differences in body weight between MC4R F1 progeny and controls generated in 2018, although there was a positive relationship between zygosity and growth. F1 homozygous/bi-allelic mutants were 30% larger than F1 heterozygotes at market size grown in earthen ponds ($p=0.022$).

Channel catfish have limited ability to synthesize n-3 fatty acids, due to a lack of elongases and desaturases. The ccβA-msEvol2 transgene containing masu salmon, *Oncorhynchus masou*, elongase gene driven by the common carp, *Cyprinus carpio,* beta-actin promoter was inserted into the channel catfish melanocortin-4 receptor (MC4R) gene using the

2-Hit 2-Oligo with Plasmid (2H2OP) method. SgRNA one (MC4R-A) targeting exon one of the MC4R gene resulted in a knockout mutation rate of 92% with 69% homozygosity/bi-allelism, a knock-in rate of 54% and a simultaneous knockout/knock-in rate of 49%. Evol2/MC4R fish were 41.81% larger than controls at 6-months post hatch (p=0.005). There was no significant increase in overall omega three content, however observed mean eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) content were 92.16% (p=0.203) and 32.82% (p=0.380) higher, respectively, in Evol2/MC4R fish than controls.

Combining ability for body weight of fingerlings grown in aquaria and food size fish grown in earthen ponds generated from channel catfish females and blue catfish, *I. furcatus*, males was calculated using a 6 x 6 diallel design. At 14-months post hatch, and a mean weight of 9.69g±8.78g, the GCA_D, GCA_S and SCA were 4.5%, 8.4% 20.8% of total variance, respectively. At 40-months, and a mean weight of 836g±250.71, the GCA_D, GCA_S and SCA for the interspecific matings were 1.1%, 0.0 and 4.6% of total variance, respectively. The heaviest and lightest families at 40-months post hatch were not the same as those at 14-months post hatch, indicating genotype-environment interactions.

Keywords: Channel catfish, *Ictalurus punctatus*, blue catfish, *Ictalurus furcatus*, myostatin, melanocortin-4 receptor, elongase, omega-3 fatty acid, growth, gene editing, transgenesis, CRISPR/Cas9, heritability, combining ability, growth, breeding, factorial design

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Chapter 1: Comparison of Genetic Programs Used for Growth Enhancement

Aquaculture continues to lead as the fastest growing food production sector at 5.8 percent annual growth (FAO, 2020). In 2018, aquaculture production was 82 million tonnes, and valued at 250 billion USD (FAO, 2020). Aquaculture has finally overtaken capture fisheries both in total value and as the main producer of fish for human consumption (FAO, 2020). Global per capita fish consumption increased by 3.1 percent since 1961, approximately double the rate of population growth (1.6 percent). Aquaculture currently employs over 20 million people worldwide, helping to lift countless people out of poverty and reduce food scarcity (FAO, 2020). China continues to be the largest fish producer, accounting for 35 percent of global fish production, with the rest of Asia making up another 34 percent (FAO, 2020). Finfish production dominates the industry accounting for 54 million tonnes, with the majority (47 million tonnes) coming from inland aquaculture (FAO, 2020).

The United States aquaculture sector produced just 468 thousand tonnes of aquatic animals, including 181 tonnes of bivalves (FAO, 2020). Catfish remains the major fish produced in the United States, accounting for approximately 70 percent of total freshwater production at approximately 136 million kg in 2014 (Torrans and Ott, 2018) down from its peak at 300 million kg in 2003 (Hanson and Sites, 2015). The United States has become the largest importing market for seafood after the European Union (FAO, 2020), with imports making up approximately 90 percent of seafood consumption and 80 percent of catfish consumption, largely from Asia (NOAA, 2020 (Hanson and Sites, 2015). This has led to a seafood trade deficit of 17 billion USD (NOAA, 2020). In order to restore the catfish industry and reduce our reliance on foreign goods, a shift

towards a more advanced production system is necessary. This will include farm management technologies, improved nutrition, disease management and genetic enhancement.

Aquaculture, while dating back to 6,000 BC in China is still a very new industry commercially when compared to terrestrial livestock production (Harland, 2019). While many societies transitioned from hunting to farming livestock during the Neolithic Revolution around 10,000 years ago, wild stocks made up the vast majority of fish consumption until the last decade (MacHugh and Bradley, 2001; FAO, 2020). Capture fisheries produced less than 20 million tonnes of fish worldwide in 1950, then rose rapidly due to industrialized fishing fleets and a growing demand, before stabilizing between 86 million tonnes and 93 million tonnes from the late 1980's to today (FAO, 2020). That plateau was largely caused by declining wild fish stocks coinciding with a rapid increase in aquaculture from less than 10 million tonnes worldwide in 1980 to 82 million tonnes today (FAO, 2020). Commercial aquaculture is a decades old industry aided by the modern technology but thousands of years behind terrestrial livestock in terms of selection, although fish have characteristics that allow rapid closure of this gap. With the use of new genetic enhancement techniques, the large genetic diversity in still available in wild and domestic stocks, and the inherent efficiencies of fish as a protein source, aquaculture has huge potential to benefit from genetic technologies in a short period of time. Unlike poultry and livestock, fish are ectothermic, live in an aquatic environment with less gravitational force, and have higher yields of meat per total body mass, making fish much more efficient at converting feed into edible meat (Tlusty et al., 2018). Advances in genetics will help aquaculture supply the increasing demand for meat in a growing and ever industrializing human population.

Traditional genetic enhancement in agriculture included various forms of selection. While limited to phenotypic observational traits with high heritability, enormous advances have been achieved. The cows, swine, chickens and other livestock and poultry are vastly superior to their wild ancestors for producing meat and other animal byproducts. Broiler chicken growth rates increased 400 percent from 1950 to 2005 due to improvements in both genetics and culture techniques (Zuidhof et al., 2014). With advances in the understanding of genetics and new technologies, greater results can be achieved in a much shorter time period. These techniques include selection, marker-assisted selection, intraspecific crossbreeding, hybridization, polyploidy, sex reversal, transgenesis and gene-editing, all of which will be discussed below.

Growth and feed conversion efficiency are the most fundamental and economically important traits affecting production, variable costs and profitability. Feed is typically the largest variable cost in aquaculture, accounting for 60 percent of operational costs in catfish farming (Goddard, 2012; Robinson and Li, 2015). Feed also represents a major contributor to greenhouse gas emissions in aquaculture, making feed conversion optimization essential for profitable and sustainable fish culture. (Hasan and Soto, 2017). Growth rate and feed conversion efficiency are highly correlated, particularly when comparing fast growing to slow growing genotypes (Dunham 2011).

Selection

Selection is a process used to improve desirable quantitative traits in a population over multiple generations. Selection of common carp, *Cyprinus carpio*, has been performed for centuries (Gjedrem and Rye, 2016). The first planned selection experiments for fish involved

resistance to endemic furunculosis in brook trout, *Salvelinus fontinalis*, (Embody and Hyford, 1925). Embody and Hyford were able to achieve an average increase in survival of 22% per generation over three generations.

Selection begins as soon as a population is brought into captivity, favoring individuals more fit for that environment, and skewing the population towards domestication. Even without direct selection, channel catfish showed an increased growth rate of 3 to 6 percent per generation due to domestication (Dunham, 2011). The fastest growing strain of channel catfish, the Kansas strain, is also the oldest domesticated strain, having been captured from the Ninnescha River in Kansas 109 years ago (Dunham, 2011). Quantitative traits are controlled by many loci and display a continuous range in phenotypic variation (Dunham, 2011). Selection is a genetic enhancement program that utilizes retaining the best performing individuals or families for a trait for brood stock to obtain genetic gain. Selection response is determined by selection intensity, phenotypic variation of the population and heritability (Dunham, 2011).

Understanding heritability of a trait is essential in selection programs as it describes how much of the phenotype can be explained by additive genetic variation, the genetic component that is mostly responsible to selection response. A trait with low heritability will be difficult to select for as its phenotype is largely determined by factors other than additive genetic variation. There are two types of heritability. Broad-sense heritability is the total genetic variation as a proportion of the total phenotypic variation (Lush, 1949). This includes effects due to dominance and epistasis. Narrow-sense heritability is the proportion of additive genetic variation in the total phenotypic variation and is the best predictor of selection response (Lush, 1949; Dunham, 2011).

In a practical sense, narrow-sense heritability measures the amount of phenotypic differences between parents that can be passed on to their offspring (Lush, 1949). Heritability measurements range from 0 to 1, where 0 indicates none of the phenotypic variation is due to genetic variation, while 1 indicates all of the phenotypic variation is due to genetic variation. High narrow-sense heritability is generally considered to be above 0.35, indicating that selection will likely be effective, while low heritability is below 0.15, indicating other genetic enhancement programs may be more successful (Dunham, 2011). Heritability varies between populations, strains and generations as the allele frequencies change over time and differ among and within populations (Dunham, 2011).

There are two major types of selection. Mass selection, also known as individual selection, involves the selection of individuals based on their own performance while family selection chooses individuals based on the mean performance of their families. Mass selection is useful due to its simplicity, requiring minimal facilities, less data collection and has lower expenses (Dunham, 2011). The major disadvantage is the possibility of inbreeding, especially with smaller populations, due to the lack of pedigree information. Family selection, while requiring more resources has greater potential for genetic gains and allows you to select for traits such as carcass traits and sex specific traits (Dunham, 2011). Combined selection, utilizing both mass and family selection, has advantages of both programs and produces the fastest and largest genetic improvements (Dunham, 2011).

Genetic enhancement for fish has a major advantage over other animals. Unlike cows, pigs, chickens and other domesticated livestock whose wild ancestors are either extinct or hybridized

with their domestic cousins, nature is still a vast source of wild populations of fish with large genetic variability. Additionally, fish typically have short generation intervals and a large number of offspring. This allows for much more complex breeding programs and more intense selection intensity. Additionally, aquatic organisms are more genetically plastic than warm blooded animals, allowing for genetic enhancement programs not possible for warm blooded animals. Despite this, as of 2010, it is estimated that only 8.2% of the world's total aquaculture production involved species developed using selection (Gjedrem and Rye, 2016). However, a much greater proportion is genetically enhanced.

Growth is the most extensively studied trait in fish. Growth has been selected for in *Oreochromis spp., Salmo solar, Oncorhynchus mykiss, O. kisutch, Cyprinus carpio, Sparus aurata, Dicentrarchus labrax, Ictalurus punctatus*, and many others. While there is variation between studies, typically there is a positive response to selection for growth averaging 7-14 percent per generation (Gjedrem and Thodesen, 2005; Dunham, 2011). Therefore, it is possible to double the growth of a population in only six generations of selection, as shown in Nile tilapia, *Oreochromis niloticus*, and Atlantic salmon, *Salmo salar* (Gjedrem and Rye, 2016).

Heritability varies among strains, populations and generations. In a (1983a) study by Dunham and Smitherman, in which three strains of channel catfish, *Ictalurus punctatus*, were selected for body weight, the strains with more recent domestication as well as higher heritability had significantly greater gains. Rio Grande, Marion, and Kansas strains, each domesticated 10 years, 20 years and 60 years respectively, had increases in body weight of 17, 18 and 12 percent respectively after one generation (Dunham and Smitherman, 1983a). Realized heritability was

0.5±0.13, 0.24±0.06, and 0.33±0.10 for Marion, Rio Grande, and Kansas respectively. In a study by Dunham and Brummet (1999), two generations of selection for growth on two strains of channel catfish, resulted just 11.5 percent gain, with no improvement in the second generation, and a cumulative heritability of 0.16 (Dunham and Brummet, 1999).

While it is possible to reach a selection plateau, in which genetic gain per generation reaches near zero or decreases, there are many examples of continued steady improvement after many generations. In a four generational study selecting for body weight in tra catfish, *Pangasianodon hypophthalmus*, average body weight increased 9.3 percent per generation (Vu et al., 2019). This large increase over four generations was possible due to a narrow-sense heritability of 0.34 (Vu et al., 2019). Increased body weight was genetically correlated with other beneficial traits, resulting in increases in standard length (3%), filet yield (1.6%), condition index (2.7%) and survival (7.4%) (Vu et al., 2019). Similarly, in a ten-generation study on GIFT Nile tilapia, *Oreochromis niloticus*, average body weight at harvest increased 107 percent over ten years, averaging 11.9 percent per generation (Hamzah et al., 2014). Another study of *O. niloticus* over two generations found average body weight gain of 24.8 percent, with the first-year gain of 34.7 percent and heritability estimate of 0.6 (Charo-Karisa et al., 2006).

Marker Assisted Selection

Marker-assisted selection (MAS) is the use of molecular or biochemical genetic markers associated with specific traits to select for individuals in a breeding program. These include traditional markers such as isozymes, RFLPs and mtDNA analysis and more powerful DNA markers including RAPD, microsatellites (SSRs), AFLPs, ESTs and SNPs (Dunham, 2011). MAS

is particularly useful for traits that are difficult or expensive to measure and have low heritability as well as sex related and carcass traits.

Isozymes are multiple forms of an enzyme, which vary in amino acid sequence, but catalyze the same reaction. They are useful in that they are directly related to performance and are inherited co-dominantly, which allows distinguishing between homozygotes and heterozygotes (Dunham, 2011). Isozymes can be separated through electrophoresis using starch gel, polyacrymalide, agarose, cellulose acetate or Cellogel (Giles and Ruddel, 1973). Hallerman et al. (1986), discovered isozymes have been linked to growth in channel catfish.

Restriction Fragment Length Polymorphism (RFLP) uses restriction endonucleases to cut DNA at restriction sites and expose mutations at or between the restriction sites. These products can be separated on an agarose gel. RFLP probes are then hybridized with the product to produce DNA fingerprints (Dunham, 2011). RFLP is tedious and so is rarely used today. Gross and Nilsson (2011) found a RFLP at the growth hormone 1 gene in Atlantic salmon, although the polymorphism was not associated with change in body weight.

Mitochondrial DNA (mtDNA) is useful in tracking the maternal lineage and traits. Mitochondrial DNA is approximately an order of magnitude more prone to mutation than genomic DNA, due to a lack of repair mechanisms during replication (Liu and Cordes, 2004). This makes it a useful tool when other markers are unavailable and with more recent evolutionary mutations (Dunham, 2011). A major advantage of mtDNA over other markers is that it does not require prior molecular information (Liu and Cordes, 2004). Because mtDNA is only inherited through the dam,

the mtDNA is only a single locus and the population structures and phylogenies may not coincide with the nuclear genome due to gender biased migration (Birky et al., 1989) or introgression (Chow and Kishino, 1995; Liu and Cordes, 2004). The mitochondrion is the main location of aerobic cellular respiration, and therefore plays an important role in growth and feed conversion. Ferguson and Danzmann (1999) found growth variation between mtDNA haplotypes in Ontario rainbow trout, *Onchorhynchus mykiss*, showing potential for MAS. Mitochondrial DNA markers have also been used to study stock structure in American eels, *Anguilla rostrate*, (Avise et al., 1986), bluefish, *Pomatomas saltatrix*, (Graves et al., 1992), red drum, *Sciaenops ocellatus*, (Gold et al., 1993), snappers, family Lutjanidae, (Chow, 1993) and sandbar sharks, *Carcharhinus plumbeus* (Heist and Gold, 1999)(Liu and Cordes, 2004).

Randomly Amplified Polymorphic DNA (RAPD) technique uses short primers to randomly bind to sites during PCR. By scanning the entire genome, the primers are able to detect large numbers of polymorphisms that will form bands if less than 3000 bp apart and can be separated by gel electrophoresis (Dunham, 2011). While very useful in genotyping, disadvantages include non-specific amplification (false positive) and inability to distinguish zygosity (Dunham, 2011). Ahmed et al. (2004) showed a large number of RAPD markers in various tilapia species with significant growth variation, although low levels of RAPD polymorphism exists between channel catfish strains, showing it is very species specific (Liu et al., 1998a).

Microsatellites, also known as simple-sequence repeats (SSRs), are short tandem repeats of 1-6bp. Major advantages of microsatellites are they are highly polymorphic, evenly distributed in genomes and they are inherited co-dominantly (Dunham, 2011). The major disadvantage is that

they are extremely time consuming for library construction, screening, designing primers and they require sequencing (Liu et al., 1998a). The catfish myostatin gene, that plays a major role in skeletal muscle growth contains several microsatellites (Kocabas et al., 2002).

Amplified Fragment Length Polymorphisms (AFLPs) are PCR-based markers able to rapidly produce hundreds of highly replicable markers (Mueller and Wolfenbarger, 1999). Two restriction enzymes (EcoRI and MseI) digest genomic DNA, oligonucleotide adaptors ligate to the fragment ends and these restriction fragments are selectively amplified using specific primer combinations (Vos et al, 1995; Dunham, 2011). These sets of restriction fragments can then be separated by gel electrophoresis without knowing the nucleotide sequence. This method is extremely useful in genotyping due to its high number of markers, high replicability, relatively, speed and ease of use. The major disadvantages are that AFLP markers are dominantly inherited and require expensive equipment. A study by Liu et al. (1998b) looking at AFLP markers found that intraspecific polymorphism was low among channel catfish and blue catfish strains while interspecific polymorphism was high between the two species. Multiple AFLP markers associated with increased growth rates were found in the F2 and backcross hybrids between channel catfish and blue catfish.

Single Nucleotide Polymorphisms (SNPs) are single base mutations, either a substitution or indel. SNPs are typically identified in genome-wide association studies (GWASs) and require sequencing. In the past this was a limiting factor, but with advances in genomics, SNPs are becoming the gold standard for markers. SNPs are normally distributed throughout a genome, occur once every 76 to 2000 nucleotides and have co-dominant inheritance. (Liu, 2007). Channel

catfish has a SNP every 76 bases on average (Dunham, 2011). The channel catfish myostatin gene has many SNPs as well as microsatellites (Kocabas et al., 2002).

Genomic selection is a type of index selection utilizing whole genome quantitative trait loci (QTL) maps and linkage disequilibrium (Goddard and Hayes, 2007). While molecular markers have been used by breeders for decades, linkage maps consisted of only a few hundred markers at most and did not fully represent the desirable traits (Illumina, 2016). The technological improvements and reduced cost of next-generation sequencing (NGS) has led to much more precise analysis and a subsequent surge in genomic sequencing. The recent developments in genomic selection allow for the development of new marker-based models for genetic evaluation and novel breeding programs that allow for increased genetic gain of complex traits per unit time and cost (Bhat et al., 2016). SNP markers and the reduction of the cost of SNP analysis were key to the commercial genomic selection to allow adequate coverage of the genome necessary for accurate genomic estimated breeding values. Genomic selection is now routine for plant breeding and is becoming more common in fish species. Over 1000 molecular markers have been mapped in channel catfish, many of which are related to growth and feed conversion efficiency (Liu and Cordes, 2004, Dunham, 2011; Li et al., 2018). Ning et al. (2018) located 22 SNPs in a 1Mb region on linkage group 5 that are significantly associated with body weight in channel catfish. Most research so far has been theoretical; however, simulations and early experimental results indicate that breeding values can be accurately predicted with genetic markers alone (Goddard and Hayes, 2007). With the improvements in accuracy and reduced cost of genomic sequencing genomic selection will likely play a major role in the future of breeding.

Intraspecific Crossbreeding

Intraspecific crossbreeding is a short-term genetic enhancement program in which individuals from two or more families or strains are bred to obtain heterosis (Dunham, 2011). The genetic mechanism behind crossbreeding is dominance, epistasis and overdominance with the goal of producing offspring with superior performance to either parent (Dunham, 2011). There are a variety of crossbreeding programs including two-breed cross, backcross, three-breed cross and recurrent selection. Success is based on combining ability, in which certain families or strains combine better than others, thereby achieving greater heterosis. Reciprocal crossbreeds typically have different performance traits, particularly with regards to growth rate (Dunham, 2011). In general, intraspecific crossbreeding improves growth in about 25% of crosses, with ranges of 10-50% improvement (Dunham, 2011). Typically, due to inherent improvements associated with domestication, domestic x domestic crosses have better performance than crosses involving wild strains (Dunham, 2011).

Dunham and Smitherman (1998b) found that six of nine (67%) channel catfish crossbreeds mad from P1 generations showed heterosis for body weight, averaging 10.3 percent above both parents. Marion female x Kansas male was the fastest growing to fingerling size (Dunham and Smitherman, 1998b). Studies on tilapia species show variable results. A diallele crossbreeding study with three strains of Nile tilapia concluded specific heterosis for F-1 crossbreeds of Stirling x Korean, Stirling x Local and Korean x Local was 7.61, 5.63 and 7.70 for body weight (Marengoni and Onoeue, 1998). However, none of the F1 crossbreeds had higher average body weight than purebred Stirling and the average of all crossbreeds was lower than that of all purebreds, indicating no gain in performance. On the other hand, a study of GIFT Nile tilapia founders using an 8 x 8

diallele cross, found that 7 of 22 crosses performed better than the best pure strain, with the largest body weight gain being 11 percent (Eknath et al., 1998). Heterosis for growth has been observed in rainbow trout, common carp, silver barb, *Barbodes gonionotus*, and Chinese shrimp, *Fennropenaeus chinensis* (Bakos, 1979; Dunham and Smitherman, 1983a; Bakos and Gorda, 1995; Dunham, 1996; Bentsen et al., 1996, Tian et al., 2006).

Interspecific Hybridization

Interspecific hybridization is the successful mating between two species to produce a viable offspring. While most hybridizations do not results in heterosis, there is potential for dramatic genetic improvement. In cases where hybridization is possible, typically artificial fertilization is necessary. Similar to crossbreeding, the effect of hybridization on performance is largely dependent on the combining ability of the specific families and strains of the parents as well as their species combining ability.

Sunshine bass, a hybrid of the white bass (*Morone chrysops*) and striped bass (*M. saxatilis*), exhibits growth higher than either parent under commercial culture conditions, has high temperature tolerance, improved osmoregulation, improved disease resistance, high angling vulnerability and high survival under culture with the ability to digest soy protein in feed (Colombo et al., 1998; Smith, 1998; Dunham, 2011). Other hybrids with increased growth include crosses of black crappie, *Pomoxis nigromaculatus* and white crappie, *P. annularis* (Hooe et al., 1994), silver carp, *Hypophthalmichthys molitrix* and bighead carp, *Aristichthys nobilis*, and hybrids between the African catfish *Clarias gariepinus*, and the Vundu, *Heterobranchus longifilis/H. bisorsalis*, (Salami et al., 1993; Nwadukwe, 1995; Bartley et al., 2000).

Interspecific hybridization can also play a role in sex specific traits including fertility and sex ratios. In the case of Nile tilapia female x blue tilapia, *O. aureus,* male hybrids are fertile with salinity and cold tolerance. Crosses of certain strains produce all-male offspring, thereby improving production as males have faster growth (Lahav and Lahav, 1990; Wohlfarth, 1994; Verdegem et al., 1997).

Of the nearly 50 North American interspecific ictalurid hybrids, only the hybrid between channel catfish females and blue catfish males has greater performance for commercially valuable traits than either parents (Smitherman and Dunham, 1985).The channel catfish female x blue catfish male is considered the most successful example of interspecific hybridization, displaying significant improvement in growth, disease resistance, low oxygen tolerance, feed conversion, survival, seinability, and dressout percentage (Smitherman and Dunham, 1985; Li et al., 2004). However, as the two species do not mate, and male blue catfish must be sacrificed in order to extract sperm, reproduction remains a bottleneck in hybrid production, although channel catfish female x blue catfish male hybrids still account for over half of all catfish raised in the United States (Hanson and Sites, 2015).

Polyploidy

Polyploidy refers to individuals with one or more extra sets of chromosomes. Most fish are naturally diploid, having a duplicated set of homologous chromosomes in somatic cells (Piferrer et al., 2009). There are some modern natural exceptions and polyploidy seems to have arisen

independently several times in fishes (Piferrer et al., 2009). While modern salmonids are diploid, they evolved from a tetraploid ancestor, which developed four sets of chromosomes during a replication event 25-100 million years ago (Allendorf and Thorgaard, 1984). In fact, all modern vertebrates evolved from a common tetraploid ancestor that lived 500 million years ago (Allendorf and Thorgaard, 1984).

While polyploidy is lethal in birds and mammals (Chourrout et al., 1986), triploid (three sets) fish are viable and have several potential economic benefits (Thorgaard et al., 1981). Triploid fish are often sterile due to a lack of gonad development, and show potential for increased growth, increased carcass yield, increased survival and increased flesh quality (Chourrout et al., 1986; Bye and Lincoln, 1986; Thorgaard, 1986; Hussain et al., 1995; Dunham, 1995; Dunham, 2011). Any improved growth characteristics, particularly after sexual maturity, are likely due to excess energy that would have otherwise been used for gonadal development. Triploids have a larger nucleus due to the extra set of chromosomes and subsequent larger cell size. Species, such as the stickleback and the ayu correct for the increased cell size by reducing their quantity of cells (Aliah et al., 1990; Dunham, 2011). Additionally, sterility allows for genetic containment of farmed fish. While sterile triploid females typically have low levels of sex hormones, sterile triploid male sex hormone profiles match those of diploids and they may exhibit sexual behavior and mate with females without fertilizing the eggs (Dunham, 2011). Triploid bivalves are commonly used in the industry due to their faster growth and yield (152%), greater survival (34%) and improved flavor (Degremont et al., 2012).

In fish, triploidy is induced by shocking the embryo with either temperature, hydrostatic pressure, anesthetics or chemicals to force retention of the second polar body (Thorgaard et al., 1981; Wolters et al., 1981; Chourrout and Itskovich, 1983; Benfey and Sutterlin, 1984; Chourrout, 1984; Cassani and Caton, 1986; Curtis et al., 1987; Johnstone et al., 1989; Dunham, 2011). Hydrostatic pressure is typically the most effective treatment, with the highest consistency and survival (Cassani and Caton, 1986; Bury, 1989; Dunham, 2011). The success of shock treatments depends on the time of initiation after fertilization as well as the magnitude and duration of the shock, with ideal parameters varying by the species, temperature and subsequent rate of development (Dunham, 2011). To induce triploidy, shock should be initiated during the second meiotic division, while tetraploids are best produced with shock during the first mitotic division (Dunham, 2011). Typically, the hatch rate is lower for triploids than diploid controls (Dunham, 2011). There is also typically a tradeoff between survival and percentage of triploids, with higher pressure producing a greater percentage of triploids but lower hatching success.

Polyploidy, including triploidy, in fish rarely improves growth. There is also often a reduction in tolerance to low dissolved oxygen and other survival traits. Triploidy is an effective tool for inducing sterility and therefore has many useful applications. Methods of polyploidy induction also play a key role in the success of the treatment. Certain species, such as salmonids, common carp, and in particular, bivalves, show great potential for improved growth, flesh quality and other carcass traits as well as sterility. It is therefore essential to understand the biology of your culture species and goals of your program in order to make effective use of polyploidy.

Sex Reversal

Sex reversal in fish is used to create monosex populations, which have a number of benefits in aquaculture. Sexual dimorphism for growth is common in fish and a monosex culture of the faster growing sex can greatly increase productivity. Additionally, unintentional reproduction can cause a variety of issues. Fish growth often slows down dramatically during reproduction due to resources being allocated to gonadal development and mating behavior as well as natural life cycle characteristics. Furthermore, sexual reproduction, particularly in ponds, or other culture environments where population size is not easily monitored, can lead to false estimates of fish biomass, creating to water quality issues and wasting of resources on unwanted individuals. Finally, monosex populations can be more environmentally safe as they cannot breed and spread to the surrounding area unless wild populations already exist.

There are several methods for producing monosex populations in fish, each of which has its own advantages and disadvantages. Manual separation of the sexes is the simplest method and requires the least amount of technology, but it is also the most labor intensive and time consuming. Additionally, some species show little or no sexual dimorphism. Hormonal sex reversal using oestrogens or androgens is commonly used in various applications. While genotypic sex is established at fertilization, phenotypic sex is determined later in development. The timing of phenotypic sex determination varies by species and is dependent on size more than age. Phenotypic sex is determined prior to hatch in some species, such as certain salmonids, (Goetz et al., 1979) and as late as the fingerling stage in species such as grass carp (Shelton et al., 1982). By raising the appropriate sex hormone levels during the phase of sexual differentiation, genetic sex can be overridden to produce the desired phenotypic sex. There are several androgens shown to be

effective in producing all male populations, with the most common being 17-methyltestosterone (Dunham, 1990). The most common and effective oestrogen used to produce all female populations is 3-oestradiol (Yamazaki, 1983; Dunham, 1990). These hormones can be administered through implants (Boney, 1983), by bath immersion (Yamazaki, 1983) or most commonly in feed (Shelton et al., 1978) depending on the species. Effectiveness of the treatment is largely based on timing and duration of hormone administration as well as the dosage, type of hormone, species and genetics (Dunham, 2011).

Channel catfish males grow 10-30% faster than females in certain strains (Benchakan, 1979; Dunham and Smitherman, 1984; Smitherman and Dunham, 1985; Dunham and Smitherman, 1987) so male monosex populations would be highly desirable. However, while all female populations can easily be produced using a variety of oestrogens, including 17ß-estradiol, producing all male populations has proved difficult in channel catfish (Goudie et al., 1985; Davis et al., 2000). Instead of creating phenotypic males, administration of testosterone produces mostly female populations. This is likely caused by channel catfish reacting to elevated testosterone levels by digesting and converting the chemical into oestrogen-like compounds, thereby sex-reversing genetic males into phenotypic females (Goudie et al., 1985; Dunham, 2011).

Certain countries and markets restrict hormone-treated food fish, such as the European Union and the United States. This is typically due to fears that the elevated hormone levels could cause issues in consumers or the natural environment. This fear is unwarranted however, as the hormones used in the treatment of fry will have been metabolized long before harvest. Nevertheless, alternative methods of sex reversal have been successful, with the most common

being temperature manipulation. Exposing fish to high temperatures during their critical period of sexual differentiation can cause a higher proportion of males than would be expected in certain species, including Nile tilapia (Baroiller et al., 1995a, b; Dunham et al., 2001). While male:female ratios as high as 19:1 can be achieved, 100% sex reversal has not been accomplished to date. In order to address this issue, sex reversal treatments must be combined with breeding. Combining sex reversal with genetic manipulation using progeny testing can create all male YY populations (Beardmore et al., 2001; Dunham et al., 2001), all male ZZ systems (Hopkins et al., 1979) and all female XX systems (Boney et al., 1984; Dunham, 2011).

The most commercially successful monosex system to date is all male Nile tilapia. First tilapia fry are sex-reversed to produce all female populations of XY neo-females and normal XX females. They are then progeny tested by breeding females with normal XY males to identify XY females, which produce 25% YY males, 50% XY males and 25% XX females. Male progeny are then progeny tested again to identify YY males. YY males are then mated to XX females to produce all XY males for commercial production. While this system is tedious, all male production can increase production of Nile tilapia by 50% and commercial production using this system is now commonplace (Dunham, 2011). YY male Nile tilapia have survival and fertility equal to normal XY males and this system successfully solves the problem of early sexual maturation and unwanted reproduced, while vastly increasing production (Beardmore et al., 2001; Dunham et al., 2001; Dunham, 2011).

Transgenesis

Transgenesis is the successful transfer of foreign DNA into the genome of another species. Success includes not just integration, but expression as a functional protein and inheritance to following generations. Vectors containing the transgene, most often plasmids, but also bacteriophages and cosmids, can be transferred into the host genome via microinjection, electroporation, viruses, sperm-mediated transport or gene-gun bombardment (Dunham, 2011).

In 2015, AquaBounty's AquAdvantage salmon became the first transgenic animal to be approved for human consumption by the United States Food and Drug Administration (FDA 2020). By integrating a Chinook salmon growth hormone gene into the genome of Atlantic salmon, growth rate was increased by 400-600 percent, reducing the grow-out period from three years to 18 months (Entis, 1998). The GH gene was linked to winter flounder, *Pseudopleuronectes americanus*, anti-freeze protein promoter and ocean pout terminator (Entis, 1998). Additionally, AquAdvantage salmon are all female, through gynogenesis and sex reversal, and sterile, through triploidy, reducing the possibility of genetic impact on natural populations in the event of escape (Waltz, 2016).

Growth hormone has been the most common gene used for transgenesis. Transgenic fish incorporating gene constructs encoding growth hormone exhibit 3-11-fold weight gain (Devlin et al., 1992; Du et al., 1992; Rahman et al., 1998; Gaffney et al., 2020). Wild-strain rainbow trout containing a salmon gene construct overexpressing growth hormone showed a 17.3-fold increase in weight (Devlin et al., 2001). However, the growth of the transgenic wild-strain did not surpass that of a fast growing domestic strain, nor did introducing the transgene to that same domestic

strain increase its growth rate. Transgenic Nile tilapia containing Chinook salmon, *Oncorhynchus tschawytscha*, growth hormone exhibited a 2.5-fold increase in growth compared to non-transgenic siblings (Rahman et al., 2005). Dunham et al. (1999) produced transgenic channel catfish containing salmon growth hormone that exhibited 33 percent faster growth than controls. These fish also exhibited lower predator avoidance to largemouth bass, *Micropterus salmoides* and green sunfish, *Lepomis cyanellus*, than controls, indicating that they would have poor performance in regards to fitness traits if escaped in the wild, which predicts environmental risk (Dunham et al., 1999). However, due to the potential dangers of transgenic fish escaping into the wild and outcompeting or breeding with wild populations, sterility or other reproductive barriers are currently essential. Gene-editing is one option or tool that may help accomplish this

Gene-Editing

Genome editing is the process of causing double stranded breaks (DSB) in genes of interest in order to induce mutation. Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and most recently, Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) are all new techniques for precise gene-editing. After a double stranded break, DNA can repair itself through either the high-fidelity homologous directed repair (HDR). or the more error-prone non-homologous end joining (NHEJ) (Ran et al., 2013: Dong et al., 2014). During the repair process, mutations often occur, including insertions, deletions (indels) and substitutions. Larger fragments, including entire genes may be inserted into the cut site, allowing both functional knock-out of the gene and knock-in of a transgene.

ZFNs

Zinc-finger nucleases are the first widespread gene-editing technology. ZFN work in pairs, with one binding to each DNA strand and function by linking customized zinc-finger protein (ZFP) to the cleavage domain of *FolkI* to induce a DSB at the target site (Wah et al., 1998: Porteus and Carroll, 2005; Miller et al., 2007). In order to initiate a DSB, ZFNs require nuclease hetero-dimerization of the two ZFNs to bind to adjacent half sites for DNA cleavage (Miller et al., 2007). While this allows for large cleavage specificities up to 24 bp, homodimers can also form that cleave other targets, which can cause cytotoxicity if the off-target cut was in an essential gene (Porteus and Carroll, 2005). Improvements in the *FolkI* nuclease to function as obligate heterodimers, reduces off target effects (Miller et al., 2007). Exogenous repair mechanisms can be paired with ZFNs allowing incorporation of a transgene through HDR (Miller et al., 2007). Targeting of the myostatin a gene in yellow catfish, *Pelteobagrus fulvidraco*, yielded a 0-2 percent mutation rate with large frequency of off target effects (Dong et al., 2011). In a study targeting the luteinizing hormone in channel catfish through electroporation, the overall mutation rate for three sets of ZFN was 19.7 percent (Qin et al., 2016). Due to the complexity of design, high cost, off-target effects and low mutation rate, ZFN's have been mostly replaced by TALENs.

TALENs

Transcription Activator-Like Effector Nucleases (TALENs) are endonucleases comprised of a DNA binding domain (TALE) from *Xanthomonas* bacteria and the endonuclease *FolkI* (Dong et al., 2014). In a similar mechanism to ZFN, a pair of TALENs binds to opposite DNA strands on either side of the target site and causes a DSB (Joung and Sander, 2013: Dong et al., 2014). During the repair process, mutations can occur, particularly with NHEJ, potentially leading to effective knock-out of the target gene (Joung and Sander, 2013: Dong et al., 2014). TALENs are both more

affordable than ZFNs and less prone to off target effects. In a follow up experiment by Dong et al. (2014), this time targeting MSTNb in yellow catfish, a 14 percent mutation rate was achieved using TALENs, far higher than the 0-2 percent achieved using ZFN to target MSTNa (Dong et al., 2014). In a 2015 study by Qin on channel catfish targeting reproductive genes LH, FSH and cfGnRH using TALENs, mutation rates were 44.7 percent, 63.2 percent and 52.9 percent respectively (Qin, 2015).

CRISPR/Cas9

Clustered regularly interspaced short palindromic repeats (CRISPRs) is group of DNA sequences stored in the genomes of some archaea and bacteria to defend against viruses (Doudna and Charpentier, 2014). CRISPRs are comprised of short repeating sequences of nucleotides and spacers, which contain viral DNA from previous virus attacks and serve as a memory bank to recognize the virus and aid in future immunity (Doudna and Charpentier, 2014; Sander and Joung, 2014). During future attacks, the spacer will be used as a template to transcribe CRISPR RNA (crRNA), comprised of the complementary spacer sequence and a short nucleotide repeat, which binds to the virus (Doudna and Charpentier, 2014; Sander and Joung, 2014). CRISPR associated protein 9 (Cas-9) is an endonuclease that binds to trans-activating CRISPR RNA (tracrRNA) and (crRNA), which then guide the Cas-9 to the target site where it makes a double stranded break (DSB) in the foreign DNA (Sander and Joung, 2014). To ensure that the Cas-9 protein does not cut an off target sequence or the CRISPR region in the host bacteria or archaea, the Cas-9 will only make a cut if there is a short (2-6 bp) DNA sequence, known as a protospacer adjacent motif (PAM), adjacent to the target sequence (Sander and Joung, 2014).

CRISPR/Cas9 can be used as a genome editing tool by simply changing the nucleotide sequence of the crRNA to bind to a target site on the host. A more modified version developed by Jinek et al. (2012) combines the crRNA and tracrRNA into a single guide RNA, simplifying the system to just two units, the Cas-9 protein and gRNA. By simply designing a 20 bp gRNA that compliments to your target site and inserting the Cas-9/gRNA complex into a cell, it will cause a DSB in a precise location. It is important to design a gRNA that does not show high complementarity to any other sites, or off target cuts can occur (Jinek et al., 2012; Doudna and Charpentier, 2014). Once the DNA is cut, the cell will repair the site using either NHEJ or HDR. In the case of NHEJ, nucleotides may be inserted or removed during the repair process, potentially causing frameshift mutations or amino acid substitutions that will effectively knock out the gene (Ran et al., 2013). With HDR, the cell will naturally fill the gap with a sequence of nucleotides using a short DNA template (Ran et al., 2013). By replacing the template with either double-stranded DNA with homologous arms to bind to the cut site, or single-stranded DNA oligonucleotides (ssODNs) it is possible to insert entire genes to the cut site (Ran et al., 2012; Jinek et al., 2012; Doudna and Charpentier, 2014). Unlike NHEJ, HDR is typically only utilized in dividing cells (Ran et al., 2013).

CRISPR/Cas-9 gene-editing has successfully increased body weight in a number of fish species including zebrafish, medaka, channel catfish, red sea bream, *Pagrus major*, olive flounder, *Paralichthys olivaceus*, and common carp (Zhong et al., 2016; Khalil et al., 2017; Yeh et al., 2017; Kishimoto et al., 2018; Wang et al., 2018; Kim et al., 2019). Myostatin is the most commonly targeted gene as it's disruption regularly increases body weight (Zhong et al., 2016; Khalil et al., 2017; Yeh et al., 2017; Kim et al., 2019). This includes increases of 12% in Olive Flounder at

250dph, 15.5% and 55% in Common Carp at 90dph and 30dph respectively, 52% in Zebrafish males and 52% in females at 6mpf, 30% in Channel Catfish at 40dph and 30% in Medaka at 16wph (Zhong et. al., 2016; Khalil et al. 2017; Yeh et al., 2017; Wang et al., 2018; Kim et. al., 2019). Gene-editing using CRISPR/Cas-9 allows for rapid increases in body weight in much shorter time periods than traditional selection. Furthermore, by combining multiple genetic enhancement strategies, superior growth results can be achieved.

References

Ahmed, M. M., Ali, B. A., & EI-Zaeem, S. Y. (2004). Application of RAPD markers in fish: Part I–some genera (Tilapia, Sarotherodon and Oreochromis) and species (*Oreochromis aureus* and *Oreochromis niloticus*) of Tilapia. *International Journal of Biotechnology*, *6*(1), 86-93.

Aliah, R. S., Yamaoka, K., Inada, Y., & Taniguchi, N. (1990). Effects of triploidy on tissue structure of some organs in ayu [*Plecoglossus altivelis*]. *Bulletin of the Japanese Society of Scientific Fisheries (Japan)*.

Allendorf, F. W., & Thorgaard, G. H. (1984). Tetraploidy and the evolution of salmonid fishes. In *Evolutionary Genetics of Fishes*. 1-53. Springer, Boston, MA.

Avise, J. C., Helfman, G. S., Saunders, N. C., & Hales, L. S. (1986). Mitochondrial DNA differentiation in North Atlantic eels: population genetic consequences of an unusual life history pattern. *Proceedings of the National Academy of Sciences*, *83*(12), 4350-4354.

Bakos, J. (1979). Crossbreeding Hungarian races of common carp, to develop more productive hybrids. Advances in Aquaculture. *Food Fisheries News Books, Farnham, Surrey, UK*, *1979*, 635-642.

Bakos, J., & Gorda, S. (1995). Genetic improvement of common carp strains using intraspecific hybridization. *Aquaculture*, *129*(1-4), 183-186.

Baroiller, J. F., Chourrout, D., Fostier, A., & Jalabert, B. (1995a). Temperature and sex chromosomes govern sex ratios of the mouthbrooding cichlid fish *Oreochromis niloticus*. *Journal of Experimental Zoology*, *273*(3), 216-223.

Baroiller, J. F., Clota, F., & Geraz, E. (1995). Temperature sex determination in two tilapia, *Oreochromis niloticus* and the red tilapia (Red Florida strain): effect of high or low temperature. Symposium on the Reproductive Physiology of Fish, University of Texas.

Bartley, D. M., Rana, K., & Immink, A. J. (2000). The use of inter-specific hybrids in aquaculture and fisheries. *Reviews in Fish Biology and Fisheries*, *10*(3), 325-337.

Beardmore, J. A., Mair, G. C., & Lewis, R. I. (2001). Monosex male production in finfish as exemplified by tilapia: applications, problems, and prospects. *Reproductive Biotechnology in Finfish Aquaculture*, 283-301.

Benchakan, M. (1979). *Morphometric and meristic characteristics of blue, channel, white and blue-channel hybrid catfishes*. Doctoral dissertation, Auburn University, AL, USA.

Benfey, T. J., & Sutterlin, A. M. (1984). Triploidy induced by heat shock and hydrostatic pressure in landlocked Atlantic salmon (*Salmo salar L.). Aquaculture*, *36*(4), 359-367.

Bentsen, H. B., Gjedrem, T., & Van Hao, N. (1996). Breeding plan for silver barb (*Barbodes gonionotus*) in Vietnam: individual (mass) selection to improve growth rate. Rep. No. 3. International Network on Genetics in Aquaculture, ICLARM, Manila, Philippines.

Birky, C. W., Fuerst, P., & Maruyama, T. (1989). Organelle gene diversity under migration, mutation, and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. *Genetics*, *121*(3), 613-627.

Boney, S. E., Shelton, W. L., Yang, S. L., & Wilken, L. O. (1984). Sex reversal and breeding of grass carp. *Transactions of the American Fisheries Society*, *113*(3), 348-353.

Bury, D. D. (1989). *Induction of polyploidy in percichthyid basses and ictalurid catfishes with hydrostatic pressure shocks*. Doctoral dissertation, Auburn University, AL, USA.

Bye, V. J., & Lincoln, R. F. (1986). Commercial methods for the control of sexual maturation in rainbow trout (*Salmo gairdneri R.*). *Aquaculture*, *57*(1-4), 299-309.

Cassani, J. R., & Caton, W. E. (1986). Efficient production of triploid grass carp (*Ctenopharyngodon idella*) utilizing hydrostatic pressure. *Aquaculture*, *55*(1), 43-50.

Charo-Karisa, H., Komen, H., Rezk, M. A., Ponzoni, R. W., van Arendonk, J. A., & Bovenhuis, H. (2006). Heritability estimates and response to selection for growth of Nile tilapia (*Oreochromis niloticus*) in low-input earthen ponds. *Aquaculture*, *261*(2), 479-486.

Chourrout, D. (1984). Pressure-induced retention of second polar body and suppression of first cleavage in rainbow trout: production of all-triploids, all-tetraploids, and heterozygous and homozygous diploid gynogenetics. *Aquaculture*, *36*(1-2), 111-126.

Chourrout, D., Chevassus, B., Krieg, F., Happe, A., Burger, G., & Renard, P. (1986). Production of second generation triploid and tetraploid rainbow trout by mating tetraploid males and diploid females—potential of tetraploid fish. *Theoretical and Applied Genetics*, *72*(2), 193-206.

Chourrout, D., & Itskovich, J. (1983, May). Three manipulations permitted by artificial insemination in tilapia: induced diploid gynogenesis, production of all triploid populations and intergeneric hybridization. In *International Symposium on Tilapia in Aquaculture*.

Chow, S. (1993). PCR-RFLP analysis on thirteen western Atlantic snappers (subfamily Lutjaninae): a simple method for species and stock identification. *Fish Bull*, *30*, 207-224.

Chow, S., & Kishino, H. (1995). Phylogenetic relationships between tuna species of the genus Thunnus (Scombridae: Teleostei): inconsistent implications from morphology, nuclear and mitochondrial genomes. *Journal of Molecular Evolution*, *41*(6), 741-748.

Colombo, L., Barbaro, A., Francescon, A., Libertini, A., Bortolussi, M., Argenton, F., Dalla Valle, L., Vianell, S. and Belvedere, P. (1998) Towards an integration between chromosome set manipulation, interge- neric hybridization and gene transfer in marine fish culture. In: Bartley, D.

and Basurco, B. (eds) Genetics and Breeding of Mediterranean Aquaculture Species. Cahiers Options Méditerranéennes 34, 77–122.

Curtis, T. A., Sessions, F. W., Bury, D., Rezek, M., & Dunham, R. A. (1987). Induction of polyploidy in striped bass, white bass and their hybrids with hydrostatic pressure. In *Proceedings Annual Conference Southeastern Association of Fish Wildlife Agencies*. 41, 63-69.

Davis, K. B., Morrison, J., & Galvez, J. I. (2000). Reproductive characteristics of adult channel catfish treated with trenbolone acetate during the phenocritical period of sex differentiation. *Aquaculture*, *189*(3-4), 351-360.

Dégremont, L., Garcia, C., Frank-Lawale, A., & Allen, S. K. (2012). Triploid oysters in the Chesapeake Bay: comparison of diploid and triploid *Crassostrea virginica*. *Journal of Shellfish Research*, *31*(1), 21-31.

Devlin, R. H., Yesaki, T. Y., Biagi, C. A., Donaldson, E. M., Swanson, P., & Chan, W. K. (1994). Extraordinary salmon growth. *Nature*, *371*(6494), 209-210.

Du, S. J., Gong, Z., Fletcher, G. L., Shears, M. A., King, M. J., Idler, D. R., & Hew, C. L. (1992). Growth enhancement in transgenic Atlantic salmon by the use of an "all fish" chimeric growth hormone gene construct. *Biotechnology*, *10*(2), 176-181.

Dong, Z., Ge, J., Li, K., Xu, Z., Liang, D., Li, J., ... & Cao, S. (2011). Heritable targeted inactivation of myostatin gene in yellow catfish (*Pelteobagrus fulvidraco*) using engineered zinc finger nucleases. *PloS one*, *6*(12), e28897.

Dong, Z., Ge, J., Xu, Z., Dong, X., Cao, S., Pan, J., & Zhao, Q. (2014). Generation of myostatin B knock-out yellow catfish (*Tachysurus fulvidraco*) using transcription activator-like effector nucleases. *Zebrafish*, 11(3), 265-274.

Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science*, *346*(6213).

Dunham, R. A. (1995). The contribution of genetically improved aquatic organisms to global food security. In *International Conference on the Sustainable Contribution of Fisheries to Food Security, Kyoto (Japan), 4-9 Dec 1995*.

Dunham, R. A. (1990). Production and use of monosex or sterile fishes in aquaculture. *Reviews in Aquatic Sciences*, *2*(1), 1-17.

Dunham, R. A., & Smitherman, R. O. (1984). Ancestry and breeding of catfish in the United States. *Ancestry and Breeding of Catfish in the United States.*, (273).

Dunham, R. A., & Smitherman, R. O. (1987). Genetics and breeding of catfish. *Southern Cooperative Series Bulletin (USA)*.

Dunham, R. A., Majumdar, K., Hallerman, E., Bartley, D., Mair, G., Hulata, G., ... & Hörstgen-Schwark, G. (2000). Review of the status of aquaculture genetics. In *Aquaculture in the Third Millennium. Technical Proceedings of the Conference on Aquaculture in the Third Millennium, Bangkok, Thailand*. 137-166.

Dunham, R.A. (2011). Aquaculture and fisheries biotechnology: genetic approaches, Wallingford, Oxfordshire, UK ; Cambridge, MA: CABI.

Dunham, R. A., Chitmanat, C., Nichols, A., Argue, B., Powers, D. A., & Chen, T. T. (1999). Predator avoidance of transgenic channel catfish containing salmonid growth hormone genes. *Marine Biotechnology*, *1*(6), 545-551.

Dunham, R. A., & Brummett, R. E. (1999). Response of two generations of selection to increased body weight in channel catfish, *Ictalurus punctatus*, compared to hybridization with blue catfish, I. furcatus, males. *Journal of Applied Aquaculture*, *9*(3), 37-45.

Dunham, R. A., & Smitherman, R. O. (1983a). Response to selection and realized heritability for body weight in three strains of channel catfish, *Ictalurus punctatus*, grown in earthen ponds. *Aquaculture*, *33*(1-4), 89-96.

Dunham, R. A., & Smitherman, R. O. (1983b). Crossbreeding channel catfish for improvement of body weight in earthen ponds. *Growth*, *47*(1), 97.

Eknath, A. E., Dey, M. M., Rye, M., Gjerde, B., Abella, T. A., Sevilleja, R., ... & Bentsen, H. B. (1998). Selective breeding of Nile tilapia for Asia. In *6th World Congress on Genetics Applied to Livestock Production*. 27, 89-96. University of New England Armidale, Australia.

Embody, G. C., & Hayford, C. O. (1925). The advantage of rearing brook trout fingerlings from selected breeders. *Transactions of the American Fisheries Society*, *55*(1), 135-148.

Entis, E. (1998). Aquadvantage salmon: A case study in transgenic food. *Animal Biotechnology*, 9:3, 165-170

FAO (2020). The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome. https://doi.org/10.4060/ca9229en

Ferguson, M. M., & Danzmann, R. G. (1999). Inter-strain differences in the association between mitochondrial DNA haplotype and growth in cultured Ontario rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *178*(3-4), 245-252.

FDA (2020). AquAdvantage Salmon. Retreived from https://www.fda.gov/animal-veterinary/animals-intentional-genomic-alterations/aquadvantage-salmon

Gaffney, L. P., Leggatt, R. A., Muttray, A. F., Sakhrani, D., Biagi, C. A., & Devlin, R. H. (2020). Comparison of growth rates between growth hormone transgenic and selectively-bred

domesticated strains of coho salmon (*Oncorhynchus kisutch*) assessed under different culture conditions. *Aquaculture*, *528*, 735468.

Giles, R. E., & Ruddle, F. H. (1973). Production and characterization of proliferating somatic cell hybrids. In *Tissue Culture*. Academic Press. 475-500

Gjedrem, T., & Rye, M. (2018). Selection response in fish and shellfish: a review. *Reviews in Aquaculture*, *10*(1), 168-179.

Gjedrem, T., & Thodesen, J. (2005). Selection. In *Selection and breeding programs in aquaculture*. Springer, Dordrecht. 89-111.

Goddard, M. E., & Hayes, B. J. (2007). Genomic selection. *Journal of Animal breeding and Genetics*, *124*(6), 323-330.

Goetz, F. W., Donaldson, E. M., Hunter, G. A., & Dye, H. M. (1979). Effects of estradiol-17β and 17α-methyltestosterone on gonadal differentiation in the coho salmon, *Oncorhynchus kisutch*. *Aquaculture*, *17*(4), 267-278.

Gold, J. R., Richardson, L. R., Furman, C., & King, T. L. (1993). Mitochondrial DNA differentiation and population structure in red drum (*Sciaenops ocellatus*) from the Gulf of Mexico and Atlantic Ocean. *Marine Biology*, *116*(2), 175-185.

Goudie, C. A., Khan, G., & Parker, N. (1985). Gynogenesis and sex manipulation with evidence for female homogameity in channel catfish (*Ictalurus punctatus*). *Ann Progress Rept*, *1*.

Graves, J., McDowell, J., Beardsley, A. M., & Scoles, D. (1992). Stock Structure Of The Bluefish *Pomatomus-Saltatrix* Along The Mid-Atlantic Coast. *Fishery Bulletin*, *90*(4), 703.

Gross, R., & Nilsson, J. (1999). Restriction fragment length polymorphism at the growth hormone 1 gene in Atlantic salmon (*Salmo salar L.)* and its association with weight among the offspring of a hatchery stock. *Aquaculture*, *173*(1-4), 73-80.

Hallerman, E. M., Dunham, R. A., & Smitherman, R. O. (1986). Selection or drift–isozyme allele frequency changes among channel catfish selected for rapid growth. *Transactions of the American Fisheries Society*, *115*(1), 60-68.

Hamzah, A., Ponzoni, R. W., Nguyen, N. H., Khaw, H. L., Yee, H. Y., & Nor, S. A. M. (2014). Performance of the genetically improved farmed tilapia (GIFT) strain over ten generations of selection in Malaysia. *Pertanika Journal of Tropical Agricultural Science*, *37*(4), 411-429.

Hanson, T. & Sites, D. (2015). 2014 U.S. Catfish Database. MSU AEC Information Report. Retrieved from http://www.agecon.msstate.edu/whatwedo/budgets/docs/catfish2014.pdf

Harland, J. (2019). The origins of aquaculture. *Nature Ecology & Evolution*, *3*(10), 1378-1379.

Heist, E. J., & Gold, J. R. (1999). Microsatellite DNA variation in sandbar sharks (*Carcharhinus plumbeus*) from the Gulf of Mexico and mid-Atlantic Bight. *Copeia*, 182-186.

Hopkins, K. D., Shelton, W. L., & Engle, C. R. (1979). Estrogen sex-reversal of *Tilapia aurea*. *Aquaculture, 18*(3), 263-268.

Hussain, M. G., Rao, G. P. S., Humayun, N. M., Randall, C. F., Penman, D. J., Kime, D., ... & McAndrew, B. J. (1995). Comparative performance of growth, biochemical composition and endocrine profiles in diploid and triploid tilapia *Oreochromis niloticus* L. *Aquaculture, 138*(1-4), 87-97.

Illumina. (2016). Genomic selection in Aquaculture: An overview of recent publications featuring Illumina technology. Retrieved April 15, 2021, from https://www.illumina.com/content/dam/illuminamarketing/documents/products/research_reviews/genomic-selection-in-agriculture.pdf

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science, 337*(6096), 816-821.

Johnstone, R., Knott, R. M., Macdonald, A. G., & Walsingham, M. V. (1989). Triploidy induction in recently fertilized Atlantic salmon ova using anesthetics. *Aquaculture, 78*(3-4), 229-236.

Joung, J. K., & Sander, J. D. (2013). TALENs: a widely applicable technology for targeted genome editing. *Nature Reviews Molecular Cell Biology*, *14*(1), 49-55.

Khalil, K., Elayat, M., Khalifa, E., Daghash, S., Elaswad, A., Miller, M., ... & Dunham, R. (2017). Generation of myostatin gene-edited channel catfish (*Ictalurus punctatus*) via zygote injection of CRISPR/Cas9 system. *Scientific Reports*, *7*(1), 1-12.

Kim, J., Cho, J. Y., Kim, J. W., Kim, H. C., Noh, J. K., Kim, Y. O., ... & Kong, H. J. (2019). CRISPR/Cas9-mediated myostatin disruption enhances muscle mass in the olive flounder *Paralichthys olivaceus*. *Aquaculture, 512*, 734336.

Kishimoto, K., Washio, Y., Yoshiura, Y., Toyoda, A., Ueno, T., Fukuyama, H., ... & Kinoshita, M. (2018). Production of a breed of red sea bream *Pagrus major* with an increase of skeletal muscle mass and reduced body length by genome editing with CRISPR/Cas9. *Aquaculture, 495*, 415-427.

Kocabas, A. M., Kucuktas, H., Dunham, R. A., & Liu, Z. (2002). Molecular characterization and differential expression of the myostatin gene in channel catfish (*Ictalurus punctatus*). *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, *1575*(1-3), 99-107.

Lahav M. and Lahav E. (1990) The development of all-male tilapia hybrids in Nir David. Isr. J. Aquaculture. Bamidgeh 42, 58–61.

Li, M. H., Robinson, E. H., Manning, B. B., Yant, D. R., Chatakondi, N. G., Bosworth, B. G., & Wolters, W. R. (2004). Comparison of the channel catfish, *Ictalurus punctatus* (NWAC103 strain) and the channel× blue catfish, *I. punctatus*× *I. furcatus*, F1 hybrid for growth, feed efficiency, processing yield, and body composition. *Journal of Applied Aquaculture*, *15*(3-4), 63-71.

Li, N., Zhou, T., Geng, X., Jin, Y., Wang, X., Liu, S., ... & Liu, Z. (2018). Identification of novel genes significantly affecting growth in catfish through GWAS analysis. *Molecular Genetics and Genomics*, *293*(3), 587-599.

Liu, Z. J., & Cordes, J. F. (2004). DNA marker technologies and their applications in aquaculture genetics. *Aquaculture*, *238*(1-4), 1-3

Liu, Z., Li, P., Argue, B. J., & Dunham, R. A. (1998a). Inheritance of RAPD markers in channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*), and their F1, F2 and backcross hybrids. *Animal Genetics*, *29*(1), 58-62.

Liu, Z., Nichols, A., Li, P., & Dunham, R. A. (1998b). Inheritance and usefulness of AFLP markers in channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*), and their F1, F2, and backcross hybrids. *Molecular and General Genetics MGG*, *258*(3), 260-268.

Lush, J. L. (1949). Heritability of quantitative characters in farm animals. *Hereditas*, 35(51), 356-375

MacHugh, D. E., & Bradley, D. G. (2001). Livestock genetic origins: goats buck the trend. *Proceedings of the National Academy of Sciences*, *98*(10), 5382-5384.

Marengoni, N. G., Onoue, Y., & Oyama, T. (1998). Offspring growth in a diallel crossbreeding with three strains of Nile tilapia *Oreochromis niloticus*. *Journal of the World Aquaculture Society*, *29*(1), 114-119.

Mueller, U. G., & Wolfenbarger, L. L. (1999). AFLP genotyping and fingerprinting. *Trends in Ecology & Evolution*, *14*(10), 389-394.

NOAA Fisheries. (2020, June 19). U.S. Aquaculture
Retrieved from https://www.fisheries.noaa.gov/national/aquaculture/us-aquaculture

Phelps, R. P., Hastey, R., Pendetar, A., Linley, L., Papanikos, N., & Dunham, R. A. (2007). Effects of temperature on the induced spawning of channel catfish and the production of channel× blue catfish hybrid fry. *Aquaculture*, *273*(1), 80-86.

Piferrer, F., Beaumont, A., Falguière, J. C., Flajšhans, M., Haffray, P., & Colombo, L. (2009). Polyploid fish and shellfish: production, biology and applications to aquaculture for performance improvement and genetic containment. *Aquaculture*, *293*(3-4), 125-156.

Porteus, M. H., & Carroll, D. (2005). Gene targeting using zinc finger nucleases. *Nature Biotechnology*, *23*(8), 967-973.

Qin, Z. (2015). Gene Editing of Luteinizing Hormone, Follicle-stimulating Hormone and Gonadotropin-releasing Hormone Genes to Sterilize Channel Catfish, *Ictalurus punctatus*, using Zinc Finger Nuclease, Transcription Activator-like Effector Nuclease and Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 Technologies. Doctoral dissertation, Auburn University, AL, USA.

Qin, Z., Li, Y., Su, B., Cheng, Q., Ye, Z., Perera, D. A., ... & Dunham, R. A. (2016). Editing of the luteinizing hormone gene to sterilize channel catfish, *Ictalurus punctatus*, using a modified zinc finger nuclease technology with electroporation. *Marine Biotechnology*, *18*(2), 255-263.

Rahman, M. A., Mak, R., Ayad, H., Smith, A., & Maclean, N. (1998). Expression of a novel piscine growth hormone gene results in growth enhancement in transgenic tilapia (Oreochromis niloticus). *Transgenic Research*, *7*(5), 357-370.

Rahman, M. A., Ronyai, A., Engidaw, B. Z., Jauncey, K., Hwang, G. L., Smith, A., ... & Maclean, N. (2001). Growth and nutritional trials on transgenic Nile tilapia containing an exogenous fish growth hormone gene. *Journal of Fish Biology*, *59*(1), 62-78.

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*, *8*(11), 2281-2308.

Sander, J. D., & Joung, J. K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature Biotechnology*, *32*(4), 347-355.

Shelton, W. L., Hopkins, K. D., & Jensen, G. L. (1978). Use of hormones to produce monosex tilapia for aquaculture. In *The Second International Symposium on Tilapia in Aquaculture. ICLARM Conference Proceedings*. 5(623), 10-33.

Shelton, W. L., Boney, S. P., & Rosenblatt, E. M. (1982). Monosexing grass carp by sex reversal and breeding. In *TO Robson, edi-tor. Proceedings, 2nd international symposium on herbivorous fish. European Weed Research Society, Wageningen, Netherlands*. 184-194.

Smith, T.I.J. (1988) Aquaculture of striped bass and its hybrids in North America. Aquaculture Magazine 14, 40–49.

Smitherman, R.O. and Dunham, R.A. (1985) Genetics and breeding. In: Tucker, C.S. (ed.) Channel Catfish Culture. Elsevier Scientific Publishing, Amsterdam, 283–316.

Thorgaard, G. H., Jazwin, M. E., & Stier, A. R. (1981). Polyploidy induced by heat shock in rainbow trout. *Transactions of the American Fisheries Society*, *110*(4), 546-550.

Thorgaard, G. H. (1986). Ploidy manipulation and performance. *Aquaculture*, *57*(1-4), 57-64.

Tian, Y., Kong, J., & Yang, C. (2006). Comparative growth and viability of hybrids between two populations of Chinese shrimp (*Fennropenaeus chinensis*). *Chinese Science Bulletin*, *51*(19), 2369-2374.

Tlusty, M., Tyedmers, P., Ziegler, F., Jonell, M., Henriksson, P., Newton, R., ... & Cao, L. (2018). Commentary: comparing efficiency in aquatic and terrestrial animal production systems. *Environmental Research Letters*, *13*(12).

Torrans, L., & Ott, B. (2018). Effect of grading fingerling hybrid catfish (♀ channel catfish×♂ blue catfish) on growth, production, feed conversion, and food fish size distribution. *North American Journal of Aquaculture*, *80*(2), 187-192.

Verdegem M.C.J., Hilbrands A.D. and Boon J.H. (1997) Influence of salinity and dietary composition on blood parameter values of hybrid red tilapia, *Oreochromis niloticus* x *O. mossambicus*. Aquaculture Research 28, 453–459.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T. V. D., Hornes, M., ... & Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, *23*(21), 4407-4414.

Vu, N. T., Van Sang, N., Phuc, T. H., Vuong, N. T., & Nguyen, N. H. (2019). Genetic evaluation of a 15-year selection program for high growth in striped catfish *Pangasianodon hypophthalmus*. *Aquaculture*, *509*, 221-226.

Wah, D. A., Bitinaite, J., Schildkraut, I., & Aggarwal, A. K. (1998). Structure of FokI has implications for DNA cleavage. *Proceedings of the National Academy of Sciences*, *95*(18), 10564-10569.

Wang, T., Wei, J. J., Sabatini, D. M., & Lander, E. S. (2014). Genetic screens in human cells using the CRISPR-Cas9 system. *Science*, *343*(6166), 80-84.

Wang, C., Chen, Y. L., Bian, W. P., Xie, S. L., Qi, G. L., Liu, L., ... & Pei, D. S. (2018). Deletion of mstna and mstnb impairs the immune system and affects growth performance in zebrafish. *Fish & Shellfish Immunology*, *72*, 572-580.

Waltz, E. GM salmon declared fit for dinner plates. *Nature Biotechnology.* 34, 7–8 (2016).

Wohlfarth, G. W. (1994). The unexploited potential of tilapia hybrids in aquaculture. *Aquaculture Research*, *25*(8), 781-788.

Wolters, W. R., Libey, G. S., & Chrisman, C. L. (1981). Induction of triploidy in channel catfish. *Transactions of the American Fisheries Society*, *110*(2), 310-312.

Yamazaki, F. (1983). Sex control and manipulation in fish. *Aquaculture*, *33*(1-4), 329-354.

Yeh, Y. C., Kinoshita, M., Ng, T. H., Chang, Y. H., Maekawa, S., Chiang, Y. A., ... & Wang, H. C. (2017). Using CRISPR/Cas9-mediated gene editing to further explore growth and trade-off effects in myostatin-mutated F4 medaka (*Oryzias latipes*). *Scientific Reports*, *7*(1), 1-13.

Zhong, Z., Niu, P., Wang, M., Huang, G., Xu, S., Sun, Y., ... & Wang, H. (2016). Targeted disruption of sp7 and myostatin with CRISPR-Cas9 results in severe bone defects and more muscular cells in common carp. *Scientific Reports*, *6*(1), 1-14.

Zuidhof, M. J., Schneider, B. L., Carney, V. L., Korver, D. R., & Robinson, F. E. (2014). Growth, efficiency, and yield of commercial broilers from 1957, 1978, and 2005. *Poultry Science*, *93*(12), 2970-2982.

Chapter 2: Growth and Survival of Myostatin Gene Edited Channel Catfish, *Ictalurus punctatus*

Abstract

The effects of CRISPR/Cas9 knockout of the myostatin (MSTN) gene in channel catfish, *Ictalurus punctatus*, was investigated. Three sgRNAs targeting exon 1 of the channel catfish myostatin gene were microinjected together with Cas9 protein in embryos. The resulting mutation rate, expression level, growth and disease resistance were studied. Efficient mutagenesis was achieved as demonstrated by PCR, Surveyor assay and DNA sequencing. A total of 209 fish survived microinjection over 3 years with an average mutation rate of 58%. The sgRNA with the highest mutation rate, 66%, was MSTN-2 gRNA, while the highest homozygosity/bi-allelism, 84%, was achieved by combining a mixture of all three gRNAs (MSTN-Mix). Mutation rate showed high variability between years with the lowest rate of 31% in 2017 and highest rate of 100% in 2018. Successful generation of MSTN KO F1 heterozygotes was achieved in 2019 by individually mating two pairs of control females with MSTN homozygous KO males. The offspring of both families inherited the mutation at a mean rate of 88%. Growth was generally higher in MSTN mutants when compared to controls at all life stages and in both pond and tank environments. At the small fingerling stage (10-20g), P1 MSTN mutants were 30% larger than controls. At the stocker stage (100-200g), P1 MSTN mutants were 88% larger than controls and P1 MSTN mutants reached market size 27% faster than controls. Heterozygous F1 mutants were 218% larger than controls at the stocker stage in ponds. MSTN mutants had reduced overall expression levels of MSTN when compared to controls (p=5.02e-3). Mean expression level of

MSTN was 2.90 times higher in controls than in MSTN KO F1 progeny (p=0.009). No one treatment had universally greater reduction in expression across all tissues, and no significant differences existed in expression levels of MSTN between tissues of wild-type fish, indicating MSTN expression is complex and dynamic. Expression was most reduced in muscle, spleen and heart, downregulating 5-fold, 3-fold and 7-fold, respectively. When challenged with *Edwardsiella ictaluri,* the causative agent of enteric septicemia of catfish (ESC), MSTN mutants performed equally or better than controls. There was a significant difference in mean hours to death (MHD) post-challenge between families (p=0.032). MSTN-B had significantly higher MHD compared to CNTRL-B (p=0.030). With a high mutation rate and inheritance as well as improved growth and disease resistance, the use of gene edited MSTN channel catfish would benefit commercial farms.

Keywords: Channel catfish, *Ictalurus punctatus*, myostatin, MSTN, growth, Enteric Septicemia of Catfish, gene editing, CRISPR/Cas9

1. Introduction

Myostatin (MSTN) is a member of the transforming growth factor-β (TGF- β) superfamily. It has been identified as an important gene in the regulation of skeletal muscle growth in vertebrates (Lee et al., 2001) by inhibiting myogenesis and hypertrophy (Thomas et al., 2000). Natural mutations in the gene have been associated with individuals displaying significant increases in growth and double muscling as seen in breeds of cattle, *Bos taurus*, such as the Belgian Blue and Piedmontese (McPherron et al., 1997). MSTN mutants have also been found in sheep, *Ovis aries*, goats, *Capra aegagrus hircus*, dogs, *Canis lupus familiaris*, and chickens, *Gallus gallus domesticus* (Clop et al., 2006, Mosher et al., 2007, Ye et al., 2007, Zhang et al., 2012). As such, myostatin is considered a valuable gene for increasing growth in animals raised for human consumption.

Advances in gene editing technology allows for precise deletions of genomic DNA, vastly expanding the research and commercial capabilities of the myostatin gene in a variety of animal food species. The myostatin gene is highly conserved among vertebrates (McPherron et al., 1997), however, the role of myostatin varies among different taxa (Maccatrozzo et al., 2001; Wang et al., 2018). While mammalian myostatin is regulated by a single copy of the gene and expressed exclusively in skeletal muscle, two copies of the myostatin gene have been identified in many species of teleost fish, with salmonids containing four copies due to a second duplication event. Moreover, myostatin is differentially expressed in many tissues of fish (Maccatrozzo et al., 2001; Østbye et al., 2007). Within diploid teleosts myostatin-b retains its role in muscle development while myostatin-a relates to immune function (Wang et al., 2018). Ictalurid catfish are unique in possessing only a single copy of the myostatin gene, found on chromosome 6, containing 3 exons

and most closely resembling MSTN-b in other diploid teleosts (Kocabas et al., 2002; Gregory et al., 2004; Karim et al., 2017).

Perhaps due to the variety of roles of myostatin across different taxa, its effect on growth shows high variability. Even within teleost fish, change in body weight between MSTN KO fish and wild type ranged from an 88 percent increase in spotted halibut, *Verasper variegatus*, (Li et al., 2012) to a 11 percent decrease in red sea bream, *Pagrus major*, (Kishimoto, 2018). In general, however, fish with a mutated myostatin gene show in an increase in body weight when compared to controls. This includes increases of 12% in olive flounder, *Paralichthys olivaceus*, at 250 days post hatch (dph), 15.5% and 55% in common carp, *Cyprinus carpio*, at 90dph and 30dph respectively, 52% in zebrafish, *Danio rerio*, males and 52% in females at 6mpf, 80% and 25% in medaka, *Oryzias latipes*, at 5wph and 8wph respectively, 30% in channel catfish, *Ictalurus punctatus*, at 40dph, 46% in zebrafish at 120dph and 30% in medaka at 16wph (Chisada et al., 2011; Gao et al., 2016; Zhong et. al., 2016; Khalil et al. 2017; Yeh et al., 2017; Wang et al., 2018; Kim et. al., 2019). Similar results are found in studies of other vertebrate taxa including increases in body weight of 8% in male chicken, *Gallus gallus domesticus*, at 18wph, 31% in rabbit, *Oryctolagus cuniculus*, 23% in sheep, *Ovis aries*, 15% in pigs, *Sus scrofa domesticus*, at birth, 26% in male mice, *Mus musculus*, and 29% in female mice (Guo et al., 2009; Lv et al., 2015; Wang et al., 2015; Guo et al., 2016; Lv et al., 2016; Wang et al., 2018; Kim et. al., 2020;).

Myostatin also appears to play a critical role in the immune system of teleost fish. In MSTN gene edited medaka challenged with red spotted grouper nervous necrosis virus (RGNNV), there were reduced expression levels of the interferon-stimulated genes, which is associated with viral

immune response, when compared with the wild type, and subsequent high levels of the virus copy number (Chiang et al., 2016). These same mutants were 25 percent larger than the wild type (Chiang et al, 2016). When wild-type zebrafish were stressed under high stocking density, expression of both MSTN-1 (a) and MSTN-2 (b) was significantly higher in the spleen when compared to fish in low stocking densities (Helterline et al., 2007). There was no change in expression of MSTN-1 or MSTN-2 in the muscle or brain between either density treatment (Helterline et al., 2007). The upregulation in only the spleen, the primary immune organ in fish, during stress, indicates the potential role of both MSTN genes in immune response.

In another zebrafish study in which both MSTN-a and MSTN-b were knocked out using CRISPR/Cas9, mutations in both paralogues caused a reduction in transcriptional levels of several critical immune-related genes and subsequent increased mortality when exposed to *Edwardsiella tarda* (Wang et al., 2018). After exposure to *E. tarda*, the MSTN-b, but not MSTN-a, mutants had reduced expression of the important immune-related transcription factor NF-κB, and consequently, several pro-inflammatory cytokines (Wang et al., 2018). These results indicate that while both paralogues contribute to the immune response, MSTN-b impacts the immune system through activation of the NF-κB pathway, while MSTN-a likely acts upstream of NF-κB (Wang et al., 2018).

The orange spotted grouper, *Epinephelus coioides*, like the channel catfish, appears to have only one copy of the MSTN gene, which plays a role in disease resistance (Chen et al., 2017). Infection by nervous necrosis virus (NNV) in orange-spotted grouper induced myostatin promoter activity (Chen et al., 2017). This could be either due to the direct role of MSTN in disease

resistance, or an immune response by the fish to allocate resources towards fighting infection, rather than promoting growth by upregulating MSTN. However, in a study on Japanese sea bass (*Lateolabrax japonicus*), liver isozyme activity was higher in fish fed diets of MSTN inhibitory peptides (Li et al., 2017). These findings indicate that reducing MSTN activity could improve immune capacity.

While there is high conservation in sequence and protein structure of both MSTN paralogues, this does not necessarily imply conservation of function. In mice, GDF11 is 90 percent identical to MSTN (GDF8), yet their functions are completely different with MSTN regulating skeletal muscle growth and GDF11 acts as a neurogenesis inhibitor (Wu et al., 2003; Gabillard et al., 2013). This is largely due to the variation in expression both temporally and spatially, with MSTN expressed primarily in muscle and GDF11 expressed in the brain (Wu et al, 2003; Gabillard et al., 2013). These discrepancies highlight the importance of research on commercially important species, and not relying solely on model species.

While there has been a large amount of research on the myostatin gene, its commercial use in agriculture has been mostly limited to natural mutations and selective breeding. Its limited industrial use can be attributed both to lack of technology, regulation on genetically modified organisms (GMOs), and an incomplete understanding of how the gene effects species across taxa. With the advent and advances of CRISPR/Cas9 and other gene editing technologies, commercialization is possible. In 2018, the U.S. Food and Drug Administration approved AquaBounty Technologies Inc. to raise the first genetically engineered animal (Atlantic salmon) for human consumption in the United States (Waltz et al., 2017). This event serves as a

breakthrough for a growing aquaculture industry in the United States and abroad and it is expected to open the door to more mainstream use of genetic editing and engineering in the near future. With this understanding, it is essential that we improve our understanding of commercially important genes and further develop efficient methods of genetic enhancement.

Preliminary research in Auburn's fish genetic enhancement lab demonstrates a high potential for improvement by generating myostatin knock out (MSTN-KO) lines. Our lab was the first to genome edit the channel catfish (Qin at al., 2016) and consistently achieve mutation rates of 60-100 percent using CRISPR/Cas9 (Qin 2015; Khalil et al., 2017; Elaswad et al., 2018; Qin, 2019). Myostatin gene edited channel catfish showed a 29.7 percent increase in body weight at 40 days post microinjection (Khalil et al., 2017). Histological analysis of muscle tissue showed that mutated individuals exhibited hyperplasia (33.7% increase in fiber number) and hypertrophy (2% increase in fiber diameter) of muscle fibers compared to controls (Figure 1) (Khalil et al., 2017).

Genetic redundancy, in which two or more genes share similar function, is fairly common, particularly relating to survival traits (Nowak et al., 1997; DeLuna et al., 2008; DeLuna et al., 2010). Functional knock out or reduced expression of a gene can cause an overexpression of other genes to compensate for its lack of function, especially if there is a paralogous gene (DeLuna et

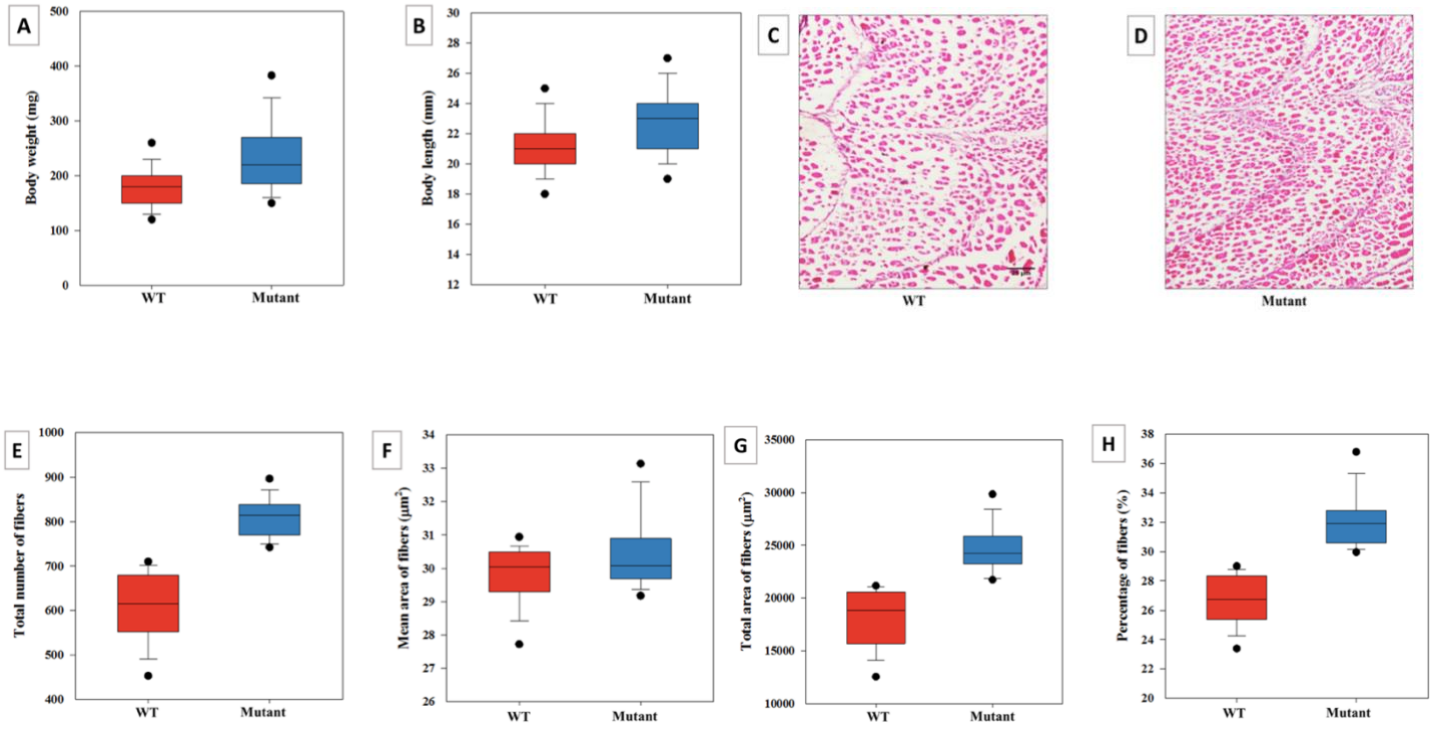


Figure 1. Evaluation of growth in myostatin (MSTN)-mutated one-month-old channel catfish fry. Body weight (**A**) and body length (**B**) of mutant (blue) and wild type (red) (n = 330). (**C**,**D**) Representative images of the ventral cross-sectional area of the epaxial muscle of wild-type (WT) (**C**) and mutant (individuals with frame-shift mutation from MSTN-Mix group) (**D**), shown by Hematoxylin and Eosin (H&E) staining. Scale bar in (**C**,**D**): 25 μm. Numbers of muscle fibers (**E**), the average area of the muscle fibers (**F**) and the total area of fibers (**G**) of mutant (blue) and wild-type (red), quantified H&E staining images (see **C**,**D**) with ImageJ. Approximately 32 stained images for each treatment were quantified. Distribution percentage of the fibers (**H**) was calculated as the total area of fibers per cross-sectional area multiplied by 100. Statistical significance was set at $p < 0.05$, and all data were presented as the mean ± standard error (*SEM*) (Khalil et al., 2017).

al., 2010). However, upregulation of a gene due to deletion of its paralog is fairly uncommon (~11%) except in the cases where overlapping function is essential for viability (DeLuna et al., 2010). In fish with multiple MSTN genes, it is possible that knock out of one gene would cause upregulation of its paralog. However, in fish with a single copy, such as ictalurid catfish, this compensatory effect may be taken up by another growth-related gene. Mutations in the SLC30A10 gene are associated with hypermagnesemia with dystonia, polycythemia and cirrhosis (HMDPC); a fatal disease due to excessive amounts of magnesium in the body (Xia et al., 2017). Zebrafish with mutated SLC30A10 genes had severe neurological and liver defects when exposed to high environmental Mn levels (Xia et al., 2017). However, overexpression of ATP2C1 (ATPase secretory pathway Ca2+ transporting 1) protected mutant embryos from Mn exposure, indicating compensatory role of ATP2C1 in the absence of SL30A10 (Xia et al., 2017). Overexpression of Hoxb4 in bone marrow cells promotes the growth of hematopoietic stem cells (HSC) (Biji et al., 2006). In homozygous Hoxb4 mutant mice, HSC levels remained normal due to compensatory up-regulation of several related Hoxb genes (Biji et al., 2006). To date, there have been no studies looking at compensatory gene effects in commercial aquaculture species. It is essential that the discipline examine expression of growth-related genes in MSTN gene edited channel catfish.

Disease resistance is a critical trait as 40% of all aquaculture production (Owens, 2012) and 40% of catfish production (Tucker, 2012) is lost to disease. Bacterial disease dominated the total number of submitted disease cases in the catfish industry in recent years (Peterman and Posadas, 2019). The most common bacterial infections in catfish are enteric septicemia of catfish (ESC) (*Edwardsiella ictaluri*), *E. pisicida,* columnaris disease (*Flavobacterium columnare*), and motile *Aeromonas* septicemia (MAS) (*Aeromonas hydrophila* and related motile aeromonads

perhaps in conjunction with *A. veronii*) (Plumb and Hanson 2010; Tekedar et al., 2013). Further improvement is needed as direct economic loss due to diseases can be extrapolated at a minimum of $60 million/yr (Zhou et al., 2018, Peterman and Posadas, 2019), and there are additional costs associated with disease management.

The objectives of this study were first to successfully knock out the myostatin (MSTN) gene in channel catfish, and then evaluate growth of the MSTN-KO fish, test inheritance of the knock-out in the F1 generations, determine their survival and disease resistance, examine gene expression and evaluate how zygosity effects growth rate. Mutation rate, growth rate, survival and gene expression at different target sites for different generations (P1 vs F1) and species were compared. Finally, disease resistance to *Edwardsiella ictaluri* (ESC) was evaluated between mutants and wild type controls.

2. Materials and Methods

All experiments were conducted at the Fish Genetics Research Unit, E. W. Shell Fisheries Research Center, Auburn University, AL. All experimental protocols used in this experiment were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC) before the experiment was initiated and followed the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) protocols and guidelines.

2.1. Design and preparation of sgRNA and CRISPR/Cas9 System

Three customized small guide RNAs (sgRNAs) were designed and generated using the Maxiscript T7 PCR-based method. First, three gene-specific oligonucleotides (MSTN1, MSTN2, MSTN3; Figure 1) containing the protospacer adjacent motif (PAM) were designed using the CRISPRscan online tool to target the channel catfish MSTN gene (GenBank Accession No. AF396747.1). Exon 1 was targeted in all four sgRNA sites in order to truncate the protein as far upstream as possible to ensure the largest knockout effect. The Universal Primer (Figure 1), containing the sgRNA scaffold was obtained from Thermo Fisher Scientific. Each oligonucleotide was reconstituted using DNase/RNase Free water to form a high stock at 10mM. To create the template for sgRNA synthesis, the three oligos were used to were synthesize double stranded DNA (dsDNA) by mixing 25µl 2x EconoTaq Plus Master Mix (Lucigen), 12.5µl Universal Primer, and 12.5µl gene-specific oligonucleotide. PCR cycling was carried out with initial denaturing at 95°C for 3 min; 5 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec with a ramp speed of -0.2°C/sec, extension at 72°C for 30 sec; 20 cycles of denaturation at 95°C for 30 sec,

Table 1. Primers used to amplify partial sequences of channel catfish, *Ictalurus punctatus*, oligonucleotides to target specified regions for CRISPR/Cas9 cleavage and single-stranded oligo donor nucleotide (ssODN). Universal primer was used to bind oligonucleotides to Cas-9 protein. Red letters indicate binding site to target gRNAs. MC4R-A, MC4R-B, MC4R-C, MC4R-A2-sense and MC4R-D-antisense were all used to target various loci in exon 1 of the channel catfish MC4R gene. MSTN-1, MSTN-2 and MSTN-3 were all used to target various loci in exon 1 of the channel catfish MC4R gene. MC4R-ALL-1F and MC4R-ALL-1R were used to amplify DNA segments flanking the target sites for guide RNAs in the catfish MC4R gene. EX1-F and EX1-R were used to amplify DNA segments flanking the target sites for guide RNAs in the catfish MC4R gene. PUC57 Vector was used to amplify a partial sequence of the Evol2 plasmid containing the elongase gene. BsalElvol2_ ssODN1 and BsalElvol2_ ssODN2 were used to flank the cut site associated with the MC4R-A2-sense gRNA to facilitate Homologous Directed Repair (HDR) in the MC4R gene. BsalElvol2_ ssODN3 and BsalElvol2_ ssODN4 were used to flank the cut site associated with the MC4R-D-antisense gRNA to facilitate Homologous Directed Repair (HDR) in the MC4R gene.

Oligo sequence (5' to 3')	Oligo name
AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	Universal Primer
taatacgactcactataGGGATGGCGCTGATCACCAGgttttagagctagaa	MC4R-A
taatacgactcactataGGGAAAGGAACTCGGAGTCgttttagagctagaa	MC4R-B
taatacgactcactataGGGCAGGATGGTGAGCGTCAgttttagagctagaa	MC4R-C
taatacgactcactataGGTGGTGGCGTTCGGTCCGAgttttagagctagaa	MSTN1
taatacgactcactataGGGCGAGGCGCAGTGTTCAGgttttagagctagaa	MSTN2
taatacgactcactataGGGTCGAGCAGCTGCTGCACgttttagagctagaa	MSTN3
GGAGATGGAGGACACGGAAG	MC4R-ALL-1F
GAGACATGAAGCAGACGCAATA	MC4R-ALL-1R
taatacgactcactataGTGATGGCGCTGATCACCAGgttttagagctagaa	MC4R-A2-sense
taatacgactcactataCGGGATGCAGCATGCACACCgttttagagctagaa	MC4R-D-antisense
taatacgactcactataCTTGTCTGTAAGCGGATGCCgttttagagctagaa	PUC57 Vector
TATCGAACGCGACAGAAACGGCTGTGATGGCGCTGATCACGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTG	BsalElvol2_ ssODN1
GCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATCAGCGGCAACCTGACCATCTCTGGAGACGTCGTGAAAAGC	BsalElvol2_ ssODN2
GAGGAGGTCTTGCGGATATGAACGTGTCGGAGCACCACGGGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTG	BsalElvol2_ ssODN3
GCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGATGCAGCATGCACACCGGAACCACAGCCTGGGCGTGCAG	BsalElvol2_ ssODN4
ACTCCTCTCTGAGACCTGAC	EX1-F
AGTTAGGCTATGAAGCAGTAGT	EX1-R
CGAAATCCGTTCCTTTTTACTG	Evol2-F
CTGGCCTGTTCCTCATGTATTT	Evol2-R

extension at 72°C for 20 sec with a ramp speed of -0.2°C/sec; and final extension at 72°C for 10 min.

The Taq Polymerase was then inactivated using 4.8µl of 0.5M ultrapure EDTA and incubated at 75°C for 20 min. The PCR product was confirmed using 1% agarose gel. The sgRNA was synthesized using the Maxiscript T7 Kit (Thermo Fisher Scientific), following the manufacture's guidelines. A mix of 2µl dH2O, 10µl dsDNA Template (created above), 2µl 10X buffer, 1µl ATP, 1µl CTP, 1µl GTP, 1µl UTP and 2µl Enzyme Mix was incubated at 37°C for 90 min. The solution was cleared of DNA contamination by adding 1µl of Turbo DNase I (Thermo Fisher Scientific), vortexing briefly and incubating at 37°C for 15 min. The magnesium ions were chelated by adding 5µl of 0.5M EDTA. The Turbo DNase I was inactivated by heating the solution at 75°C for 10 min. The obtained sgRNAs were purified using Zymo RNA Clean and Concentrator kit (Zymo Research). The sgRNAs were stored in a -80°C freezer.

The Cas9 protein was acquired from PNA Bio (3541 Old Conejo Rd, Newbury Park, CA 91320) and reconstituted in dH_20 to a concentration of 1mg/ml. Twenty minutes prior to fertilizing the eggs, three sets of injection solutions were prepared by mixing equal parts of Cas9 protein with each of the sgRNAs individually, and a fourth solution combining all three sgRNA with Cas9 protein (MSTNmix). The mixture was incubated on ice for 10 minutes before adding phenol red to a total ratio of 1:1:1 of Cas9, sgRNA and phenol red, respectively. The final concentrations of Cas9 protein and sgRNA were 300-350ng/µl and 150-200ng/µl, respectively.

2.2. Brood stock husbandry, selection and spawning

Brood stock were cultured in 0.04-ha earthen ponds averaging 1-meter in depth. They were fed a 32 percent protein catfish pellet at 1-2% of their body weight per day five days per week. Dissolved oxygen was maintained above 3 mg/L using a ½ horsepower surface aerator (Air-O-Lator).

The Kansas strain of channel catfish was chosen as broodstock due to their superior growth and fry output when induced by injection of luteinizing hormone releasing hormone analogs (LHRHa). Individuals were chosen based on their health and secondary sexual characteristics. Males with well-developed papilla and large, muscular heads that were wider than the rest of their bodies were chosen. Dark coloring and scarring from territorial fighting were also signs of quality males in reproductive condition. Females with soft, well-rounded abdomen that were wider than their head, and a swollen urogenital opening were chosen. Broodstock were minimally handled and kept in tanks for as short a time as possible to avoid stress.

Males were terminated by a percussive blow to the head followed by pithing. The body cavity was opened carefully with a scalpel, ensuring not to pierce any organs. Testes were removed using tweezers and/or scissors and washed in a weigh boat with 0.9 percent saline using bottled distilled water, removing any blood or tissue. The water was drained, and the testes weighed before macerating the testes using scissors to release sperm. The homogenized testes were then filtered into a 50mL falcon tube using a 100-micron screen. The sperm was then diluted with 0.9 percent saline solution to a maximum of 10mL/gram of testes. Sperm concentration was tested using a Nanodrop 2000 Spectrophotometer (Thermo Scientific) and

running a simple linear regression between sperm concentration and absorbance using the equation y=-2.450+0.185lnX (Adjusted R2=0.601) at 450nm (Cuevas-Uribe & Tiersch, 2011). Motility was analyzed under a 10X light microscope while sperm count was checked under 40X magnification. Ideally, sperm was used immediately, but could be stored at 4°C for one week, with declining quality or up to two weeks with the addition of gentamycin at a concentration of 1mg/mL sperm solution.

Females were weighed and gently placed in labeled 32mm mesh bags. The spawning bags were then placed in a flow through holding tank so that the fish was fully submerged, ensuring that water quality was ideal with dissolved oxygen levels above 5 mg/L. A 14-gauge implanter was loaded with 100µg/kg body weight of luteinizing hormone releasing hormone analog (LHRHa) implant. The needle was then inserted at a 45-degree angle ventrally adjacent to the pelvic fin and the implant was inserted. The ovulation time was predicted based on the degree-hours according to Phelps et al. (2007). Water temperature ranged from 26-28°C. Females were checked 36 hours after LHRHa injection and every four hours after that until ovulation. Once a female began ovulating, indicated by eggs visible on the bag, she was carefully transferred into a tank with 100 mg/L buffered tricaine methane sulfonate (MS-222) with equal parts sodium bicarbonate until completely anesthetized. Prior to this time, a thin layer of vegetable shortening, Crisco, was used to coat several 20cm diameter metal egg collection pans. The fish was then dipped in fresh water to wash off the MS-222 and gently dried with a towel. A thick layer of vegetable shortening was applied around the urogenital opening, including the pelvic fins to prevent sticking of the eggs during stripping. The female was hand stripped into the greased pan by gently applying pressure on the abdomen from the anterior end of the abdomen posteriorly towards the vent. Eggs would

usually flow freely, well rounded and golden in color, with minimal blood. The stripping ensured that the eggs could not contact freshwater, which can activate the eggs. Eggs were then covered with another spawning pan to maintain moisture and transported to the molecular laboratory.

Approximately 200-300 eggs were transferred to a greased pan for fertilization. Approximately 3mL of sperm solution was added to the eggs and mixed gently with fingertips. Fresh water was added to barely cover the eggs in order to activate the sperm and eggs and the water was swirled to form a single layer and prevent sticking. After two minutes, the eggs should be fertilized, and three more cm of water was added to the eggs and the eggs were left to harden for 15 minutes.

While the embryos were hardening, 5-10µl of the Cas9/sgRNA/phenol red mixture was loaded into 1.0mm OD borosilicate glass capillary microinjection needles using a microloader. The tips of the needles were opened by breaking the end with a scalpel. The needle was then inserted into the micropipette holder to its deepest range and tightened, ensuring a tight seal. The compressed air cylinder was opened, and pressure was adjusted to 7000kPa and 0.824 m^3/hr using the pressure regulator. The injection volume was adjusted to 50nl by manipulating the pressure, the length of injection and the needle diameter. Injection volume was measured by injecting a drop of mineral oil on a hemocytometer.

After 15 minutes 100-200 embryos were transferred in a single layer to a greased 100mm petri dish and covered with Holtfreter's solution (Table 2). The petri dish was placed on the stage of the microscope. In one smooth motion, the needle was lowered until it pierced the chorion and

yolk, and the pedal of the microinjector was depressed, delivering the CRISPR solution, and withdrawing slowly. For best results, the solution was injected as close to the blastodisc as possible. When the blastodisc was not visible, the solution was spread throughout the embryo by depressing the pedal while simultaneously withdrawing the needle smoothly. To reduce mosaicism, embryos were injected between 15 min and 90 min post-fertilization, while they remained in the one cell stage. Injection controls were injected with 50nl of phenol red, while non-inject controls were not injected.

Embryos were placed in four-liter tubs of Holtfreter's solution (Table 2) with 10 mg/L doxycycline kept at 27°C with continuous aeration. The solution was changed, and dead embryos were removed daily. After about 5 days, or when the embryos were moving rapidly within the egg membrane and close to hatch, doxycycline treatment was discontinued. At 20 dph fry were moved to aquaria in recirculating systems until large enough to be PIT (Passive Integrated Transponders) tagged and moved to earthen ponds.

2.3. Mutation Analysis

Pelvic fin-clip samples (10-20mg) were collected in sterile 1.5mL Eppendorf tubes and kept in a -80°C freezer until DNA extraction. Genomic DNA was extracted using proteinase K digestion and ethanol precipitation using the following protocol: fin clips were digested in 600µl of cell lysis buffer (100mM NaCl, 10mM Tris, 25mM EDTA, 0.5% SDS) and 2.5µl of proteinase K in a 55°C hot water bath for 4-8 hours, with occasional vortexing. Protein was precipitated by adding 200µl of protein precipitation solution (Qiagen, 19300 Germantown Road Germantown, MD 20874), vortexed, stored on ice for 12 minutes and centrifuged for 8 minutes at 15,000rcf. The

supernatant containing DNA was then precipitated with isopropanol followed by centrifugation for 5 minutes at 15,000rcf and finally washed twice with 75% ethanol by inverting gently 5-times before being dissolved in dH_2O. DNA concentration and purity was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and concentration was adjusted to 500ng/µl.

Table 2. Name, type of material, company, catalog number (if applicable) of materials and equipment used in microinjection protocol for CRISPR/Cas9 in channel catfish, *Ictalurus punctatus.*

Name of Material/ Equipment	Type	Company	Catalog Number	Comments/Description
Reproboost® implant	Hormone	Center of Marine Biotechnology		Luteinizing hormone releasing hormone analog (LHRHa) for artificial spawning
TRICAINE-S	Anesthesia	Western Chemical. Inc.		For sedation of brood stock fish during hormone injection and egg stripping.
Phenol red	Reagent	Sigma-Aldrich	P0290	0.5%, sterile filtered
Stereo microscope	Equipment	Olympus	213709	For visualizing the eggs during microinjection
Microinjector	Equipment	ASI-Applied Scientific Instrumentation	Model MPPI-3	For the delivery of the injection material into the embryos
Micromanipulator	Equipment	ASI-Applied Scientific Instrumentation	Model MM33	For holding and controlling the movement of the injection needle.
Eppendorf Microloader	Tool	Eppendorf	5242956.003	For loading injection solution into microinjection needles.
Vertical needle puller	Equipment	David Kopf Instruments	Model 720	For pulling microinjection needles
Borosilicate glass capillaries	Tool	Fisher Scientific		1 mm outer diameter (OD), for making microinjection needles.
Petri dish	Tool	VWR	25384-302	For holding the embryos during the microinjection.
Crisco®	Vegetable shortening	The J.M. Smucker Company		For coating spawning pans and petri dishes.
Holtfreter`s solution	Reagent	Lab Made		59 mM NaCl, 0.67 mM KCl, 2.4 mM $NaHCO_3$, 0.76 mM $CaCl_2$, 1.67 mM $MgSO_4$ (Armstrong et al., 1989) to incubate the microinjected embryos till hatch.
Doxycycline hyclate USP (monohydrate)	Antibiotic	Letco Medical	690904	Added to Holtfreter's solution to 10 ppm to prevent bacterial infections.

The primer set EX1-F, EX1-R (Table 1) was designed using Primer3plus to encapsulate all possible mutation sites (Figure 1). The Expand High Fidelity^{PLUS} PCR System (Roche) was used with 500ng of genomic DNA. A Bio-Rad T100 Thermal Cycler was used to run PCR with an initial denaturing at 95°C for 3 min; 34 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 40 sec with a ramp speed of -0.2°C/sec, extension at 72°C for 40 sec; and final extension at 72°C for 10 min.

The PCR product was confirmed on a 1% TAE Tris base, acetic acid and EDTA agarose gel. The PCR product from the treatment fish was mixed with PCR product from a wild type control of the same family at a 1:1 ratio. The combined product was then hybridized in a BioRad Thermocycler using an initial denaturing at 95°C for 3 min; 85°C for 1 min with a ramp speed of -0.2°C/sec, 75°C for 1 min with a ramp speed of -0.2°C/sec, 65°C for 1 min with a ramp speed of -0.2°C/sec, 55°C for 1 min with a ramp speed of -0.2°C/sec, 45°C for 1 min with a ramp speed of -0.2°C/sec, 35°C for 1 min with a ramp speed of -0.2°C/sec, 25°C for 1 min with a ramp speed of -0.2°C/sec.

The Surveyor® mutation detection kit (Integrated DNA Technologies) was used to detect mutations. Hybridized products were mixed with Nuclease S, Enhancer S, MgCl2 and Reaction Buffer (2) according to kit instructions and incubated at 42°C for one hour. The digested products were separated on a 1.5 percent TBE (Tris borate EDTA) agarose gel and compared with that of control samples.

To confirm and identify the mutations, positive samples were sequenced using the TA cloning method. The largest individuals from each treatment that repeatedly were confirmed as mutants using Surveyor Analysis were sequenced. First, genomic DNA from three mutants per treatment was amplified with PCR using Expand High Fidelity^{PLUS} PCR System (Roche) using the above protocol. The PCR product was verified using a 1 percent TAE agarose gel and cloned into the TOPO® TA Cloning® Kit (Invitrogen) with 20 clones per sample and sent to MCLabs (320 Harbor Way, South San Francisco, CA 94080) for sequencing. The resulting sequences were interpreted using the MAFFT sequence alignment tool.

To test for mosaicism and relative gene expression in different tissues, three fish from each treatment group were sacrificed at 200 days post hatch (dph) and muscle, spleen, heart, liver, brain and gonad samples were collected in 1.5mL tubes and immediately submerged in liquid nitrogen. All samples were stored at -80°C until RNA extraction. Samples were ground up using a mortar and pestle in the presence of liquid nitrogen. RNA was extracted using TRIzol reagent following the manufactures protocol. The quality and concentration of each sample was tested using gel electrophoresis and NanoDrop 2000 spectrophotometer (Thermo Scientific). All samples had an A260/280 ratio above 1.8 and were diluted to 500ng/µl.

All RNAs were reverse-transcribed to complimentary DNA (cDNA) using the iScript Synthesis Kit (Thermo Scientific). Each reaction had a total volume of 10.0µl, comprised of 4.0µl iScript reaction mix, 1.0µl iScript reverse transcriptase, 500ng RNA template and water to reach a total value of 10.0µl. The solution was then brought to 25°C for 5 min, 42°C for 30 min, and 85°C and 5 min using a Bio-Rad T100 Thermal Cycler.

Quantitative real time PCR (qRT-PCR) was performed on a Bio-Rad C1000 Thermal Cycler using SsoFast EvaGreen supermix kit (Thermo Scientific) following the manufacturer's protocol. All reactions were performed in a 10.0µl total reaction volume comprised of 1.0µl of each primer (5µ mol/l, 5.0µl SsoFast EvaGreen supermix, 2.0µl RNase/DNase-free water and 1.0µl 200ng/µl cDNA. qRT-PCR cycling was carried out with initial denaturing at 95°C for 3 min; 40 cycles of denaturation at 95°C for 5 sec and fluorescence measurement at 57°C for 5 sec, followed by 65°C for 5 sec and 95°C with a ramp speed of -0.1°C/sec to calculate the melting curve.

The mRNA levels of each sample were then normalized to the levels of 0hpf PGC marker-gene expression for the control or to the control sample for the same time point for knockdown effect measurement. Ribosome 18S mRNA was used as the reference gene. Crossing-point (Ct) values were then exported into an Excel spreadsheet from the Bio-Rad CRX Manager program. The relative expression ratio of a target gene to the 18S ribosomal RNA gene was analyzed for significance using a randomization test in the REST software (http://rest.gene-quantification.info/), assuming 100% quantification efficiency.

2.4. Generation of F1 progeny

Two and three-year old P1 myostatin gene edited channel catfish with good secondary sexual characteristics were chosen as brood stock. In 2019, only two Kansas strain gene edited males were sexually mature, and each was paired with a wildtype Kansas female in 70-liter glass aquaria. Two pairs of wildtype Kansas strain channel catfish from the same family were paired in

identical conditions as controls. The system received flow-through water from a source pond between 26-28°C. Dissolved oxygen was maintained above 5 mg/L using an air stone diffuser. Both males and females were injected with 100µg liquid LHRHa/kg BW adjacent to the pelvic fin. Starting 24 hours after injection, the bottom of the aquaria were checked every two hours for courtship behavior and egg masses. Egg masses were weighed and transferred to 4L bins of Holtzfreter's solution and maintained according to the protocol in 2.2.

2.5. Grow out and growth sampling

One-hundred fry per genetic type were stocked into 3-replicate 50L aquaria in recirculating systems for growth experiments. Fish in each aquarium were fed ad-libitum with Aquamax powdered and pelleted fish diets and catfish diets.

Pellet feed size was adjusted to a maximum of ¼ the size of the mouth as the fish grew. Fry were fed Purina® AquaMax® powdered starter feed until they were large enough to eat Purina® AquaMax® 100. Both feeds contained 50% protein. All fish were fed every day to satiation. Mutants and controls generated in 2017 were kept separately in 50L aquaria until 12-months post-hatch, when they were pit-tagged and transferred to a 0.04ha earthen pond and kept communally with channel catfish, blue catfish, and hybrids to bring the density to a commercial level of 10,000 fish/ha. Fish were fed daily to satiation. P1 mutants and controls generated in 2018 were kept separately in 50L aquaria until 12-months post hatch, when they were pit tagged and transferred to three 200L communal recirculating system (RAS) tanks. The experimental fish were kept communally with channel catfish, blue catfish, and hybrid catfish, at a density of 2 fish/L and fed daily to satiation. F1 mutants and controls generated in 2018 were kept separately in 50L

aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 0.04ha earthen pond and kept communally with channel catfish, blue catfish, and hybrids to bring the density to a commercial level of 10,000 fish/ha and fed daily to satiation. P1 mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 200L communal flow through tank with unrelated channel catfish, blue catfish, *I. furcatus*, and channel catfish x blue catfish hybrids at a density of 2 fish/L and fed daily to satiation. F1 mutants and controls generated in 2019 were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 0.04ha earthen pond and kept communally with channel catfish, blue catfish, and hybrids to bring the density to a commercial level of 10,000 fish/ha and fed daily to satiation. Fifteen fish per genetic type remained in the aquaria in the recirculating system. The fish were sampled at 6-month intervals in both aquaria and ponds. During these growth trials any mortality due to disease was identified by family, genotype and sex, and the pathogen identified.

2.6. Enteric Septicemia of Catfish (ESC) Challenge

The *E. ictaluri* isolate S97-773 was provided by the USDA-ARS, Aquatic Animal Health Research Unit, Auburn, AL and stored as a glycerinated stock at -80°C until thawed for use. The stock was inoculated in 50mL brain heart infusion (BHI) broth with 131 mg/L deferoxamine (DFO) and grown on a stir plate at 28°C for 24-48 hours, depending on growth. Cultures were expanded into 150L and then 2L BHI and DFO broth, by adding 20mL and 100mL suspensions respectively, and grown under the same conditions for about 24 hours each expansion until the cell density reached 1 x 10^8 cells/mL based on the absorbance value read at 600nm (OD_{600}). The average number of colony forming units per milliliter (CFU/mL) of bacterial cells used to

challenge the fish was determined using standard plate counts run in triplicate. Confirmation of *E. ictaluri* was determined using an analytical profile index (API) 20E test (BioMérieux).

A total of 104 F1 heterozygous MSTN mutant channel catfish, *Ictalurus punctatus*, in two families (MSTN-A and MSTN-B) and 24 control channel catfish from two families were challenged with *E. ictaluri*. All water was treated and discharged into a septic system to eliminate all pathogens. Fish were acclimated in dechlorinated tap water at 27°C for 48 h prior to experiment. Each family was divided evenly into four 365 L tanks (height, width and length were 57 cm, 72 cm and 3 m, respectively) with continuous aeration and individual sand bio-filters. Three tanks were used as identical treatment tanks (infected) and one as control. Throughout the experiment, water temperature was 28.0 ± 0.5°C, pH was 7.0 ± 0.2, dissolved oxygen was 6.70 ± 0.40 mg/L, nitrite was <0.50mg/L, alkalinity was 80 mg/L and ammonia concentration was <0.50mg/L.

Before starting the challenge, water was lowered in each tank to 100 L and biofilters were turned off. One liter of *E. ictaluri* bacterial suspension containing approximately 1.0×10^8 cells/mL was added to each treatment tank resulting in a final concentration of ~1.0×10^6 cells/mL. The control tank received 1 L of Tryptic soy broth (TSB) only. Fish were immersed statically for one hour with aeration. After one hour, water was added to a final volume of 250 L and the biofilters were turned on.

Fish were observed every 12 hours and dead or moribund fish were removed and sampled. Moribund fish were necropsied to confirm the cause of death. Kidney and liver samples were taken from fish to confirm *E. ictaluri* as the causative agent of mortality. Moribund fish were euthanized

with 300 ppm buffered MS-222 according to IACUC protocols. Dissolved oxygen and temperature were measured twice daily. Fish were not fed the day before or the day of the challenge but were fed *ad libitum* the day following infection and for the remainder of the experiment. Survivors and mortalities were genotyped as needed. The challenge was continued for one week until 100% mortality. Outgoing water was detoxified with 20 ppm Clorox.

2.8. Statistical analysis

To calculate differences in body weight between MSTN mutants and controls, a paired t-test was performed using R programming language (R Core Team, Vienna, Austria). To calculate differences in body weight between multiple gRNAs, NIC and IC, as well as between F1 MSTN families and CNTRL families a one-way ANOVA and Tukey's multiple comparisons test were performed using R programming language. In cases where different treatments were kept in separate aquaria at varying densities, a regression based on density was calculated, and weights were adjusted accordingly before running the statistical analysis. Differences in mutation rate were calculated with logistic regression using R programming language. To calculate differences in mortality rate at specific time points and at the end of the ESC challenge, mean hours to death (MHD) and cumulative percent mortality (CPM) were analyzed using paired t-test, one-way ANOVA and Tukey's multiple comparisons test on R programming language. To calculate the rate of disease progress, area under the mortality progress curve (AUMPC) was used in conjunction with ANOVA. This measurement was adapted from the index of area under wilt progress curve (AUWPC) commonly used in crop research to evaluate quantitative disease resistance of different cultivars and measure disease severity (Jeger and Viljanen-Rollinson, 2001). In this study, AUMPC was calculated using the following formula:

$$\text{AUMPC} = \sum_{i=1}^{n-1} [(y_i + y_{i+1})/2] (t_{i+1} - t_i)$$

where y_i is the number of moribund fish and dead fish at time t_i and n is the total number of time points. AUMPC index of each family at each time point was calculated with cumulative mortality data. ANOVA was used to compare AUMPC indices between treatments at each time point.

3. Results

3.1. Growth

A total of 8 MSTN mutant P1 channel catfish and 31 control channel catfish generated in 2017 were PIT tagged and transferred into a 0.04-ha earthen pond at 1-year post hatch (mean body weight for MSTN and CNTRL were 58.26g and 46.90g, respectively). At 12-months post hatch, MSTN mutants had an observed mean 25% and 11.36g larger than controls, but not significantly ($p= 0.328$, $r^2= 0.029$) (Table 3). At 18-months post hatch and just reaching the lower end of market size, MSTN mutants had an observed mean 44% and 182.41g larger than controls ($p=0.036$, $r^2=0.1203$) (Figure 1). At 24-months post hatch, the fish were at standard market size, and MSTN mutants had an observed mean 27% and 249.17g larger than controls, but not significantly ($p= 0.096$, $r^2= 0.1545$). At 31-months post hatch, the observed mean for MSTN mutants was 25% and 358.00g larger than controls, but not significantly ($p=0.191$, $r^2= 0.054$). At 36-months post hatch, the fish were near oversize for processor acceptability, and MSTN mutants had an observed mean 10% and 176.00g larger than controls ($p= 0.043$, $r^2= 0.058$).

A total of 10 MSTN mutant P1 channel catfish and 27 control channel catfish were generated in 2018. At 6-months post hatch, the observed mean of MSTN mutants was 5% and 0.47g smaller than controls, but not significantly ($p= 0.675$, $r^2= 0.005$) (Table 4). At 18 months post hatch, MSTN mutants were 93.5% and 110.18g larger than controls ($p=3.34e-6$, $r^2=0.447$) (Figure 2). At 30-months post hatch, MSTN mutants were 177% and 629.38g larger than controls, but not significantly ($p= 0.639$, $r^2= 0.0002$).

Table 3. Mean body weights (grams), sample size (N), standard deviation (SD) and coefficient of variation (CV) of P1 myostatin (MSTN) knockout mutant and control channel catfish, *Ictalurus punctatus*, generated in 2017, at 12-months, 18-months, 24-months, 31-months and 36-months post hatch. Mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 0.04ha earthen pond and kept communally with unrelated channel catfish, blue catfish, *I. furcatus*, and hybrids to bring the density to a commercial level of 10,000 fish/ha and fed daily to satiation. Sampling was performed with partial seining of the pond. Paired t-test was used to determine significant p-values (<0.05)

Mean Weight (grams)

	12 months				18 months				24 months				31 months				36 months			
	N	Mean	SD	CV	N	Mean	SD	CV	N	Mean	SD	CV	N	Mean	SD	CV	N	Mean	SD	CV
Control	29	46.90	23.45	50.01	29	417.59	217.48	52.08	16	917.50	212.78	23.19	15	1412.00	368.90	26.13	8	1820.00	305.75	16.80
MSTN	5	58.26	24.71	42.42	8	600.00	169.79	28.30	3	1166.67	299.56	25.68	5	1770.00	177.20	10.01	5	1996.00	473.79	23.74
p-value		0.3280				0.0360				0.1545				0.1914				0.043		

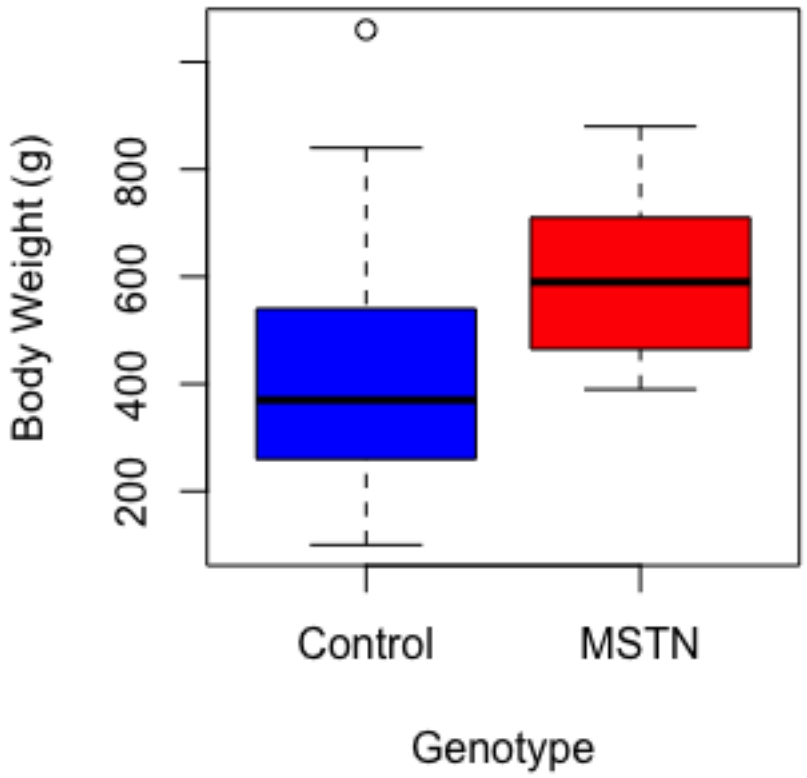


Figure 2. Box and whisker plot of body weights (grams) of control (blue) and P1 myostatin (MSTN) knockout (red) channel catfish, *Ictalurus punctatus*, generated in 2017, at 18-months post hatch. Mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 0.04ha earthen pond and kept communally with unrelated channel catfish, blue catfish, *I. furcatus*, and hybrids to bring the density to a commercial level of 10,000 fish/ha and fed daily to satiation. The plot shows median of the data (dark line), the lower and upper quartiles (25% and 75%; top and bottom of the box respectively), the area 1.5 times the interquartile range (whiskers), and outlier (circle). MSTN mutants (N=8) were 44% and 182.41g larger than controls (N=29) ($p=0.036$, $r^2=0.1203$).

Table 4. Mean body weights (grams), sample size (N), standard deviation (SD) and coefficient of variation (CV) of P1 myostatin (MSTN) knockout mutant and control channel catfish, *Ictalurus punctatus*, generated in 2018, at 6-months, 18-months and 30-months post hatch. Mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 200L communal flow through tank with unrelated channel catfish, blue catfish, *I. furcatus*, and channel catfish x blue catfish hybrids at a density of 2 fish/L and fed daily to satiation. Sampling was performed with partial seining of the pond. Paired t-test was used to determine significant p-values (<0.05).

	Mean Weight (grams)											
	6 months				18 months				30 months			
	N	Mean	SD	CV	N	Mean	SD	CV	N	Mean	SD	CV
Control	27	9.07	2.34	25.74	31	117.82	36.61	31.07	10	356.45	198.78	55.8
MSTN	10	8.6	4.46	51.91	8	228	89.02	39.05	6	985.83	310.51	31.5
p-value		0.675				3.34E-06				6.39E-01		

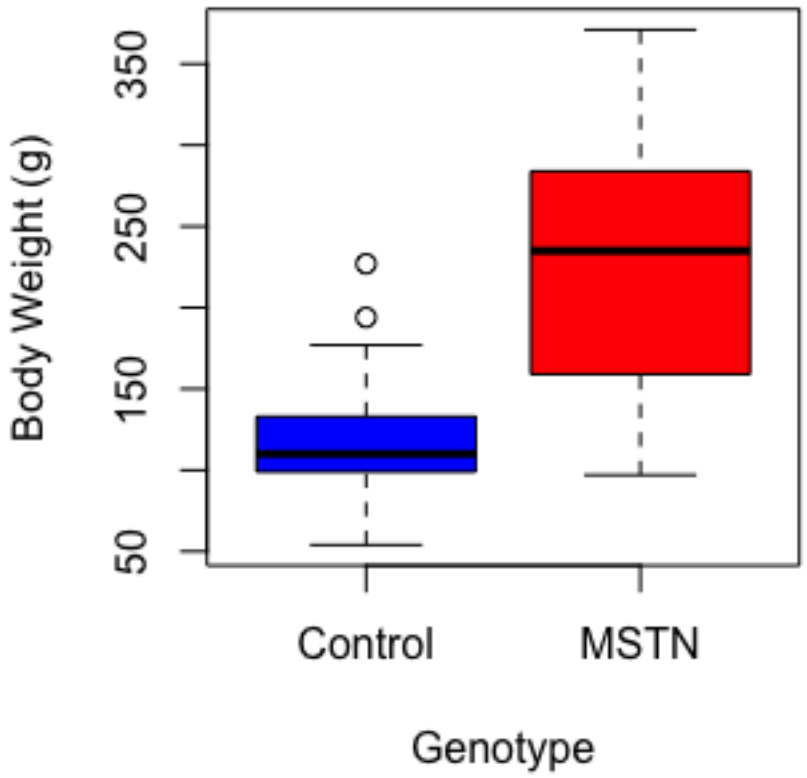


Figure 3. Box and whisker plot of body weights (grams) of control (blue) and P1 myostatin (MSTN) knockout (red) channel catfish, *Ictalurus punctatus*, generated in 2018, at 18-months post hatch. Mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 200L communal flow through tank with unrelated channel catfish, blue catfish, *I. furcatus*, and channel catfish x blue catfish hybrids at a density of 2 fish/L and fed daily to satiation. The plot shows median of the data (dark line), the lower and upper quartiles (25% and 75%; top and bottom of the box respectively), the area 1.5 times the interquartile range (whiskers), and outliers (circles). MSTN mutants (N=8) were 93.5% and 110.18g larger than controls (N=31) ($p=3.34e-6$, $r^2=0.447$).

A total of 104 MSTN mutant P1 channel catfish and 22 control channel catfish were generated in 2019. Three different cut sites in exon 1 were targeted with sgRNAs (MSTN-1, MSTN-2, MSTN-3) as well as a mix of all three sgRNA's (MSTN-Mix) a control injected with just phenol red dye (IC) and a non-injected control (NIC). Mutants and controls were kept separately in 50L aquaria until 6-months post hatch when they were large enough to be PIT tagged and moved to a 200L communal recirculating aquaculture system (RAS) tank. They were kept at a density of 2 fish/L and fed daily to satiation. There were differences among treatments at 6-months post hatch (p=0.0036). At 6-months post hatch, the observed mean for Inject-Controls was 10% and 1.33g smaller than Non-Inject Controls, but not significantly (p=0.655) (Table 5). MSTN-1 mutants were 47% and 6.33g larger than Non-Inject Controls (p=0.001). MSTN-2 mutants were 31% and 4.15g larger than Non-Inject Controls (p=0.015). The observed mean for MSTN-3 mutants was 5% and 0.69g larger than Non-Inject Controls, but not significantly (p=0.735). The observed mean for MSTN-Mix mutants was 20% and 2.77g larger than Non-Inject Controls, but not significantly (p=0.128). Overall, at 6-months post hatch MSTN mutants were 30% and 4.03g larger than controls (p= 0.004, r^2= 0.062).

There were differences in body weight among treatments at 12-months post hatch (p=0.0003). At 12-months post hatch, Inject-Controls were 42% and 44.94g larger than Non-Inject Controls, but not significantly (p=0.095) (Table 5). Observed mean of MSTN-1 mutants was 8% and 9.83g larger than Non-Inject Controls, but not significantly (p=0.563). MSTN-2 mutants were 38% and 41.25g larger than Non-Inject Controls (p=0.007). MSTN-3 mutants were 14% and 15.54g larger than Non-Inject Controls, but not significantly (p=0.397). Observed mean of MSTN-Mix mutants was 21% and 19.11g smaller than Non-Inject Controls, but not significantly

Table 5. Mean body weights (grams), sample size (N) standard deviation (SD) and coefficient of variation (CV) of P1 myostatin (MSTN) knockout mutant and control channel catfish, *Ictalurus punctatus*, generated in 2019, at 6-months, 12-months and-18 months post hatch. Three different cut sites in exon 1 were targeted with sgRNAs (MSTN-1, MSTN-2, MSTN-3) as well as a mix of all three sgRNA's (MSTN-Mix) a control injected with just phenol red dye (IC) and a non-injected control (NIC). Mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 200L communal flow through tank with unrelated channel catfish, blue catfish, *I. furcatus*, and channel catfish x blue catfish hybrids at a density of 2 fish/L and fed daily to satiation. ANOVA was used to determine significant p-values (<0.05) indicated by asterisk.

Mean Weight (grams)

		6 months				12 months				18 months			
		N	Mean	SD	CV	N	Mean	SD	CV	N	Mean	SD	CV
sgRNA	NIC	17	13.529	3.75	28	17	108.06	36.1	33	17	435.35	140	32
	IC	5	12.2	2.28	19	5	153	46.5	30	3	540.33	249	46
	MSTN-1	22	*19.864	8.46	43	22	117.89	50.2	43	22	466.36	207	44
	MSTN-2	41	*17.683	6.34	36	41	*149.3	68.1	46	36	495.39	205	41
	MSTN-3	16	14.219	3.72	26	16	123.59	52	42	14	436	152	35
	MSTN-Mix	25	16.296	4.73	29	25	88.944	32.4	36	25	410.08	135	33
Genotype	Control	22	13.227	3.47	26	22	118.27	42.1	36	20	451.1	156	35
	MSTN	104	*17.259	6.35	37	104	123.53	59.1	48	97	457.76	183	40

(p=0.241). Overall, at 12-months post hatch MSTN mutants were 4% and 5.26g larger than controls, but not significantly (p= 0.693, r^2= 0.001).

There were no significant differences in body weight between treatments at 18-months post hatch (p=0.468) (Table 5). At 18-months post hatch, Inject-Controls were 24% and 104.98g larger than Non-Inject Controls, but not significantly (p=0.349) (Figure 3). MSTN-1 mutants were 7% and 31.01g larger than Non-Inject Controls, but not significantly (p=0.591). MSTN-2 mutants were 14% and 60.04g larger than Non-Inject Controls, but not significantly (p=0.255). MSTN-3 mutants were 0.65g larger than Non-Inject Controls, but not significantly (p=0.992). MSTN-Mix mutants were 6% and 25.28g smaller than Non-Inject Controls, but not significantly (p=0.651). Overall, at 18-months post hatch MSTN mutants were 1% and 6.66g larger than controls, but not significantly (p= 0.880, r^2= 0.0002).

A total of 71 control female x MSTN male heterozygous MSTN mutant F1 channel catfish in two families (MSTN-A and MSTN-B) and 60 control channel catfish in two families (CNTRL-A and CNTRL-B) were generated in 2019. Significant differences in body weight existed among treatments at 6-months post hatch (p=5.35e-14). At 6-months post hatch, CNTRL-B was 70% and 6.22g smaller than CNTRL-A (p=3.32e-5) (Table 6). MSTN-A mutants were 36% and 5.46g larger than CNTRL-A (p=8.34e-5). MSTN-B mutants were 25% and 3.77g larger than CNTRL-A (p=0.010). Overall, at 6-months post hatch MSTN mutants were 65% and 7.86g larger than controls (p= 9.18e-12, r^2= 0.304).

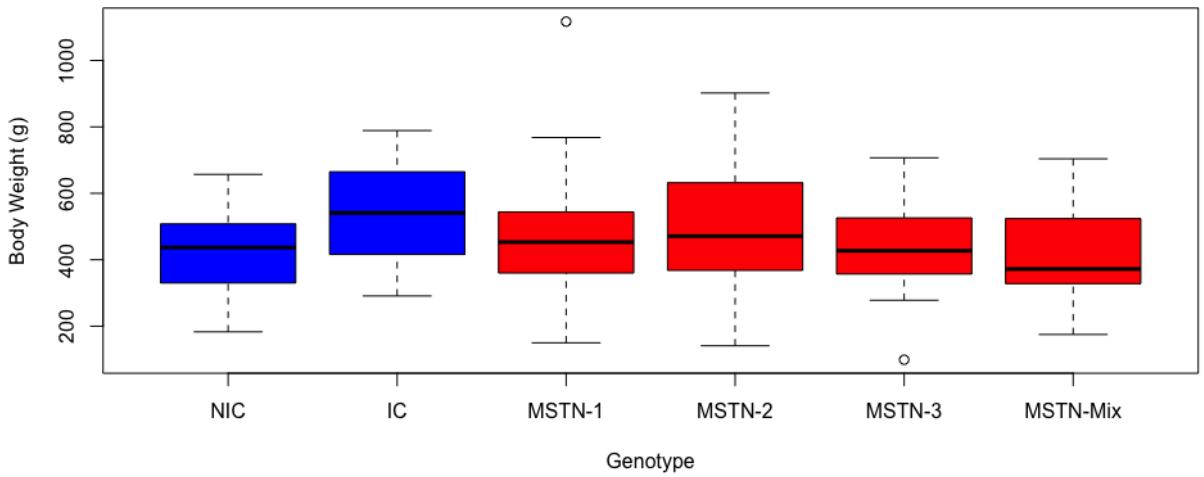


Figure 4. Box and whisker plot of body weights (grams) of control (blue) and P1 myostatin (MSTN) knockout (red) channel catfish, *Ictalurus punctatus*, generated in 2019, at 18-months post hatch. Three different cut sites in exon 1 were targeted with sgRNAs (MSTN-1, MSTN-2, MSTN-3) as well as a mix of all three sgRNA's (MSTN-Mix) a control injected with just phenol red dye (IC) and a non-injected control (NIC). Mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 200L communal flow through tank with unrelated channel catfish, blue catfish, *I. furcatus*, and channel catfish x blue catfish hybrids at a density of 2 fish/L and fed daily to satiation. The plot shows median of the data (dark line), the lower and upper quartiles (25% and 75%; top and bottom of the box respectively), the area 1.5 times the interquartile range (whiskers), and outlier (circle). Overall, at 18-months post hatch MSTN mutants were 1% and 6.66g larger than controls ($p= 0.880$, $r^2= 0.0002$, $N=117$).

Table 6. Mean body weights (grams), sample size (N), standard deviation (SD) and coefficient of variation (CV) of F1 heterozygous myostatin (MSTN) knockout mutants and control channel catfish, *Ictalurus punctatus*, at 6-months, 9- months and 12-months post hatch. Two families of MSTN F1 progeny (MSTN-A and MSTN-B) and two families of wild-type (CNTRL-A and CNTRL-B) were generated. Mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 0.04ha earthen pond and kept communally with unrelated channel catfish, blue catfish, *I. furcatus*, and hybrids to bring the density to a commercial level of 10,000 fish/ha and fed daily to satiation. Sampling was performed with partial seining of the pond. ANOVA was used to determine significant p-values (<0.05) indicated by asterisk.

		Mean Weight (grams)											
		6 months				9 months				12 months			
		N	Mean	SD	CV	N	Mean	SD	CV	N	Mean	SD	CV
Family	MSTN-A	41	*20.598	7.02	34	41	*24.11	8.1	34	36	*113.06	41.1	36.3
	MSTN-B	30	*18.9	6.85	36	29	*23.034	8.63	37	29	*113.15	36.9	32.6
	CNTRL-A	30	15.133	4.34	29	30	17.233	5.46	32	30	46.7	35.5	76
	CNTRL-B	30	8.9167	1.79	20	29	10	2.07	21	30	24.367	9.01	37
Treatment	MSTN	71	*19.88	6.95	35	70	*23.664	8.28	35	65	*113.1	38.9	34.4
	CNTRL	60	12.025	4.55	38	59	13.678	5.5	40	60	35.533	28	78.9

Significant differences in body weight existed among treatments at 9-months post hatch (p=5.36e-14). At 9-months post hatch, CNTRL-B was 72% and 7.23g smaller than CNTRL-A (p=6.99e-5) (Table 6). MSTN-A mutants were 34% and 6.88g larger than CNTRL-A (p=4.31e-5). MSTN-B mutants were 39% and 5.80g larger than CNTRL-A (p=0.001). Overall, at 9-months post hatch MSTN mutants were 72% and 9.99g larger than controls (p= 1.11e-12, r^2= 0.330).

Significant differences in body weight existed among treatments at 12-months post hatch (p<2e-16). At 12-months post hatch, CNTRL-B was 92% and 22.33g smaller than CNTRL-A (p=0.011) (Table 6; Figure 5). MSTN-A mutants were 142% and 66.36g larger than CNTRL-A (p=8.34e-13). MSTN-B mutants were 142% and 66.45g larger than CNTRL-A (p=3.45e-12). Overall, at 12-months post hatch MSTN mutants were 218% and 77.56g larger than controls (p<2.2e-16, r^2= 0.565).

3.2. Mutation analysis

A total of 209 fish survived microinjection of CRISPR/Cas9 and sgRNA targeting exon 1 of the MSTN gene over 3 years. The overall the mutation rate of the survivors was 58% (122/209) (Table 7). The highest mutation rate resulted from the MSTN-2 gRNA, although the difference was not statistically significant (p>0.05). Of the 122 P1 mutants 64% were homozygous/bi-allelic and 36% were heterozygous. MSTN-2 and MSTN-Mix led to higher levels of homozygosity/bi-allelism than other target sites (MSTN-2 p=0.014; MSTN-Mix 0.004). The average mutation rate for all F1 MSTN channel catfish was 88% (71/81). All F1 MSTN channel catfish were heterozygous.

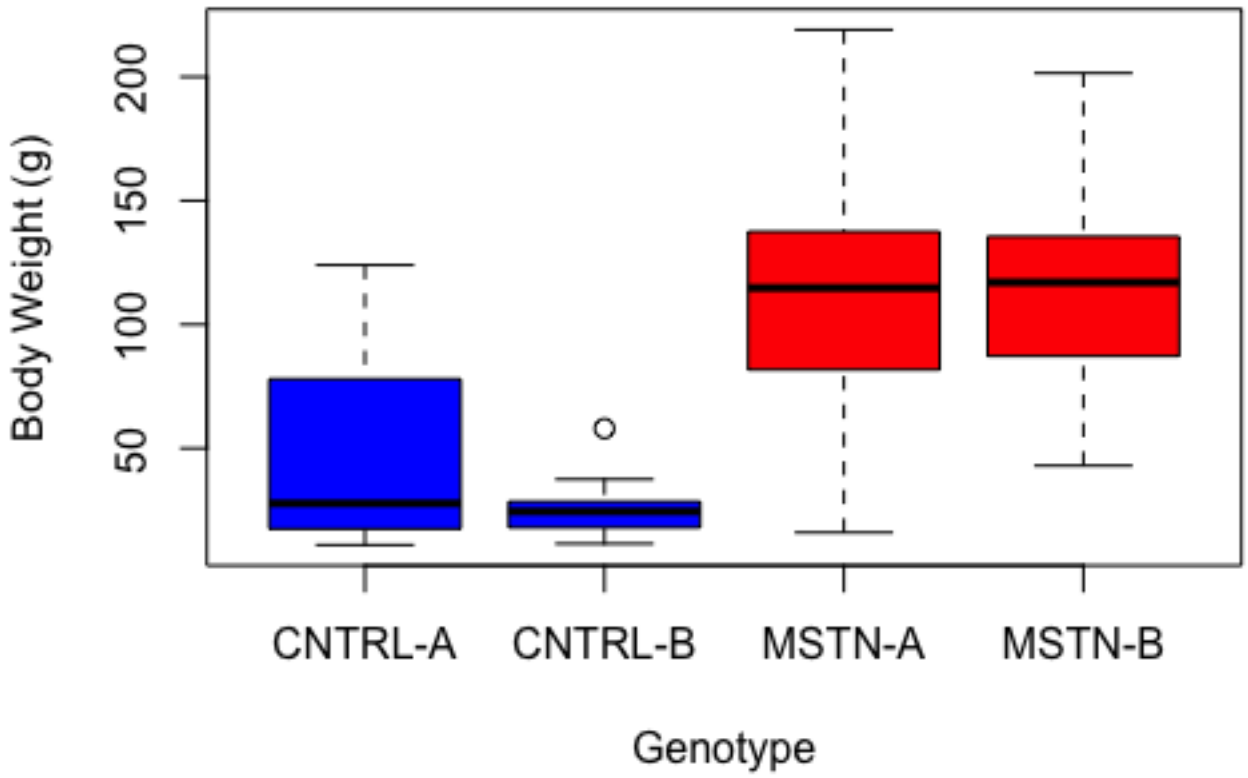


Figure 5. Box and whisker plot of body weights (grams) of Control (blue) and heterozygous F1 myostatin (MSTN) knockout (red) channel catfish, *Ictalurus punctatus*, generated in 2019 at 12-months post hatch. Two families of MSTN F1 progeny (MSTN-A and MSTN-B) and two families of wild-type (CNTRL-A and CNTRL-B) were generated. Mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 0.04ha earthen pond and kept communally with unrelated channel catfish, blue catfish, *I. furcatus*, and hybrids to bring the density to a commercial level of 10,000 fish/ha and fed daily to satiation. The plot shows median of the data (dark line), the lower and upper quartiles (25% and 75%; top and bottom of the box respectively), the area 1.5 times the interquartile range (whiskers), and outlier (circle). Overall, at 12-months post hatch MSTN mutants were 218% and 77.56g larger on average than controls ($p<2.2e-16$, $r^2= 0.565$, N=120).

Table 7. Mutation rate and zygosity of all myostatin (MSTN) gene edited channel catfish, *Ictalurus punctatus*, generated in 2017, 2018 and 2019. P1 indicates fish gene edited through microinjection using CRISPR/Cas9. MSTN-1, MSTN-2 and MSTN-3 indicate gRNA's targeting different sites on exon 1 of the MSTN gene, while MSTN-Mix was an equal combination of all three sgRNA's. Approximately 50nl of solution, composed of 300-350ng/µl Cas9 protein and 200ng/µl sgRNA, was injected into each embryo close to the blastodisc 15 min after fertilization using a glass capillary needle. F1 indicates the offspring of control channel catfish female x homozygous/bi-allelic MSTN mutant channel catfish male. Two families of F1 channel catfish (control (CNTRL)xMSTN-A and CNTRLxMSTN-B) were generated in 2019 from mutants spawned in 2017.

Year	Genotype	Mutation Rate	Homozygosity/Bi-allelism
2017	P1 MSTN Channel	31% (8/26)	63% (5/8)
2018	P1 MSTN Channel	100% (10/10)	30% (3/10)
2019	P1 MSTN-1 Channel	59% (22/37)	41% (9/22)
2019	P1 MSTN-2 Channel	66% (41/62)	73% (30/41)
2019	P1 MSTN-3 Channel	46% (16/35)	68% (11/16)
2019	P1 MSTN-Mix Channel	64% (25/39)	84% (21/25)
2019	F1 CNTRLxMSTN-A (ch)	91% (41/45)	0% (0/41)
2019	F1 CNTRLxMSTN-B (ch)	83% (30/36)	0% (0/36)

Gel electrophoresis indicated mutations in each target site in both P1 and F1 generations (Figure 6). Multiple bands in the image corresponded to expected cut sites. In most instances, 3 bands were associated with mutations while 1 band indicated a wild-type sequence, although some mutations were indicated by 5 bands, potentially indicating multiple cuts. Each positive result was confirmed with a second gel.

Sequence results confirmed mutations in each of the target sites indicated by gel electrophoresis. Each gRNA tested generated mutations in exon 1 in the MSTN gene (Figure 7). All mutations occurred outside of the target sites and each gRNA and caused deletions. There were no insertions revealed. The MSTN mutation was inherited in the offspring (F1 MSTN-B). The MSTN-Mix treatment generated the same mutation as the MSTN-3 gRNA, indicating that only one gRNA bound to the Cas9 protein. Guide RNAs MSTN-2 caused a large deletion (2181bp) outside of the target site.

3.4. Relative Gene Expression

Significant differences existed between overall expression levels of MSTN between treatments ($p=5.02e-3$) (Figure 8). Mean expression level of MSTN was 3.63 times higher in controls than in MSTN-A ($p=5.36e-3$) Mean expression level of MSTN was 2.41 times higher in controls than in MSTN-B ($p=0.036$). Mean expression level of MSTN was 2.90 times higher in controls than in combined MSTN F1 progeny ($p=0.009$). No significant differences existed in expression levels of MSTN between tissues of wild-type fish ($p=0.436$). However, no one treatment had universally greater reduction in expression across all tissues, indicating MSTN expression is complex and dynamic.

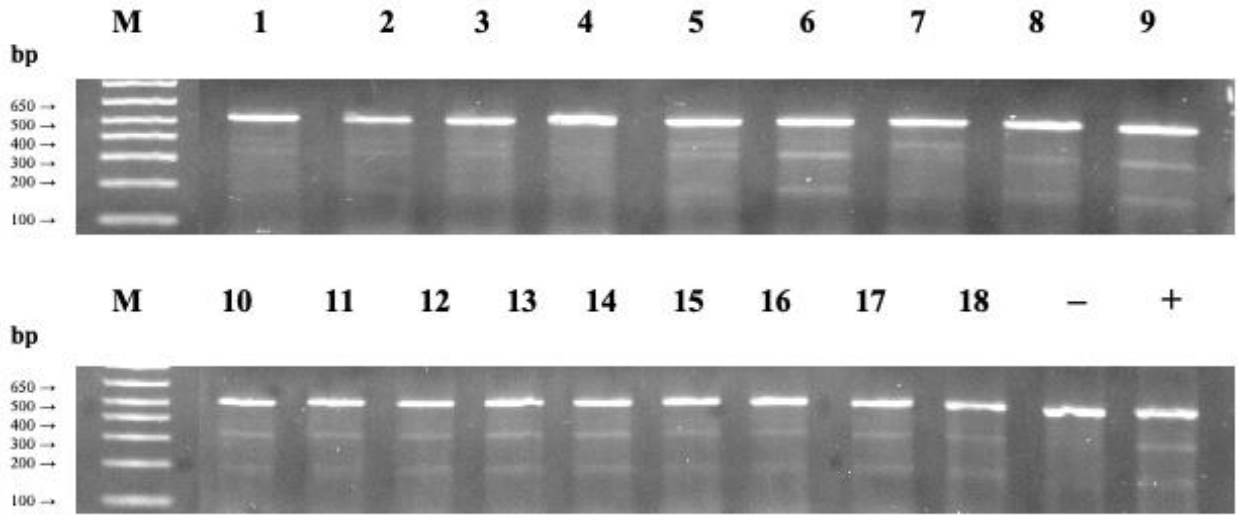


Figure 6. Identification of edited myostatin (MSTN) gene sequences in channel catfish, *Ictalurus punctatus*, using the surveyor mutation detection assay. All samples were hybridized with an equal volume of non-injected control (Sample –) to detect both homozygotes/bi-allelic and heterozygotes. Wild-type sequences are indicated with a single 482 bp band, while mutations are signified by three bands. Sample 1 came from an embryo injected with MSTN-1 gRNA/Cas9. Samples 2-4 came from embryos injected with MSTN-2 gRNA/Cas9. Samples 5-13 came from F1 progeny of CNTRL female x MSTN male mutants (MSTN-A). Samples 14-18 came from F1 progeny of CNTRL female x MSTN male mutants (MSTN-B). Sample – came from wild-type control. Sample + came from previously identified MSTN P1 mutant. M indicates 1kb marker.

Wild-Type

actcctctctgagacctgacctggctgatc**ATG**CATTTAGCGCAGGTTCTGATTTCGCTGGGCTTCGTGGTGGCGTTCGGTCCGATGGCGCGCACTGACA
CCGGAGCACCGGAGCAGCAGCAGCAGCAGCAACCTACCGCCGTGACGGAGGAGCGCGAGGCGCAGTGTTCAGCGGCCAGCGCGTGC
GCTTTCCGCCAGCACAGCAAGCAGCTCCGTCTGCAAGCCATCAAGTCCCAGATTCTGAGCAAACTGCGCCTCAAACAAGCTCCCAAC
GTGAGCCGCGATGTGGTCAAGCAGTTGCTCCCGAAAGCGCCACCGGTGCAGCAGCTGCTCGACCTGTACGACGTGCTCGGGGACGAC
GGCAAGCCGGGCACAGCGCTCCAGGACGAGGAGGAGGACGACGAGGAGCACGCCACCACCGAGACCGTCATGAGCATGGCCGCCG
AGCgtgagtccctttactactgcttcatagcctaact

MSTN-1

Wild Sequence

5’ TTCGTGGTGGCGTTCGGTCCGATGGCGCGCACTGACACCGGAGCACCGGAGCAGCAGCAGCAGCAGCAACCTACC 3’

AA: FVVAFGPMARTDTGAPEQQQQQQPT

Mutated Sequence

5’ TTCGTGGTGGCGTTCGGTCCGATGGCGCGCACTGACACCGGAGCACCGG------AGCAGCAGCAGCAGCAACCTACC 3’ [-6]

AA: FVVAFGPMARTDTGAPEQQQQPT [-2]

MSTN-2

Wild Sequence

5’ ACC//GAGCGCGAGGCGCAGTGTTCAGCGGCCAGCG// CCAACCCCACTGAACACGT 3’

AA: T// EREAQCSAAS//PTPLNT

Mutated Sequence

5’ ACC----------/----------TCAACCCCACTGAACACGT 3’ [-2184]

AA: T//-----// STPLNT [-728]

MSTN-3

Wild Sequence

5’ AAGCAGTTGCTCCCGAAAGCGCCACCGGTGCAGCAGCTGCTCGACCTGTACGA 3’

AA: KQLLPKAPPVQQLLDLY

Mutated Sequence

5’ AGCAGTT----------CGCCACCGGTGCAGCAGCTGCTCGACCTGTACGA 3’ [-11]

AA: SSPPVQQLLDLY [-7/+2]

MSTN-1,2,3

Wild Sequence

5’ TTCGTGGTGGCGTTCGGTCCGATGGCG//AGCGCGAGGCGCAGTGTTCAGCGGCCAG//CCACCGGTGCAGCAGCTGCTCGACCTGT 3’

AA: FVVAFGPMA// REAQCSAA// PPVQQLLDL

Mutated Sequence

5’ AGCAGTT----------CGCCACCGGTGCAGCAGCTGCTCGACCTGTACGA 3’ [-11]

AA: SSPPVQQLLDLY [-7/+2]

F1 MSTN-B

Wild Sequence

5’ TTCGTGGTGGCGTTCGGTCCGATGGCGCGCACTGACACCGGAGCACCGGAGCAGCAGCAGCAGCAGCAA 3’

AA: FVVAFGPMARTDTGAPEQQQQQQPT

Mutated Sequence

5’ TTCGTGGTGGCGTTCGGTCCGATGGCGCGCACTGACACCGGAGCACCGG------AGCAGCAGCAGCAGCAACCTACC 3’ [-6]

AA: FVVAFGPMARTDTGAPEQQ--QQPT [-2]

Figure 7. CRISPR/Cas9 induced mutations in exon 1 of myostatin (MSTN) gene coding sequence of channel catfish, *Ictalurus punctatus*, using various gRNAs. The exons and introns are indicated by upper and lower case and the underlined bold uppercase is the start codon. The primers used in PCR are indicated in red. The guide RNA target sites are indicated in green followed by PAM (Protospacer adjacent motif, NGG) in blue. Deletion mutations are represented by a dashed line with each dash corresponding to a nucleotide that has been deleted. Double slash indicates wild-type continuation of the sequence for simplicity. Single slash indicates that there is a large deletion. Each sequence starting with 5’ and ending with 3’ came from a single reaction representing a single allele. Brackets indicate deletion/insertion/substitution value. Wild-type sequence acquired from Genbank (Accession No. AF396747.1). Corresponding predicted amino acid sequence indicated by “AA”. Red letters indicate substitutions. Predicted amino acid sequence acquired from Expasy and confirmed with NCBI ORFfinder.

Significant differences existed between overall expression levels of MSTN in muscle tissue between treatments (p=1.91e-4) (Figure 8). Downregulation occurred in MSTN-A, MSTN-B and MSTN-2. Mean expression level of MSTN in brain tissue was 3.85 times higher in controls than in MSTN-A (p=3.37e-3). Mean expression level of MSTN in brain tissue was 3.66 times higher in controls than in MSTN-B (p=2.26e-3). Mean expression level of MSTN in brain tissue was 6.37 times higher in controls than in MSTN-2 (p=1.35e-4).

Significant differences existed between overall expression levels of MSTN in spleen tissue between treatments (p=9.97e-6) (Figure 8). Downregulation occurred in MSTN-A and MSTN-B while upregulation occurred in MSTN-2. Mean expression level of MSTN in brain tissue was 2.32 times higher in controls than in MSTN-A (p=3.17e-3). Mean expression level of MSTN in brain tissue was 4.99 times higher in controls than in MSTN-B (p=1.64e-5).

Significant differences existed between overall expression levels of MSTN in heart tissue between treatments (p=3.68e-6) (Figure 8). Downregulation occurred in MSTN-A, MSTN-B and MSTN-2. Mean expression level of MSTN in brain tissue was 9.76 times higher in controls than in MSTN-A (p=1.8e-6). Mean expression level of MSTN in brain tissue was 4.53 times higher in controls than in MSTN-B (p=1.21e-3). Mean expression level of MSTN in brain tissue was 5.80 times higher in controls than in MSTN-2 (p=2.91e-4).

Significant differences existed between overall expression levels of MSTN in liver tissue between treatments (p=6.52e-5) (Figure 8). Downregulation occurred in MSTN-A and MSTN-2

and upregulation occurred in MSTN-B. Mean expression level of MSTN in brain tissue was 2.25 times higher in control than in MSTN-A (p=1.30e-3).

Significant differences existed between overall expression levels of MSTN in brain tissue between treatments (p=1.07e-3) (Figure 8). Downregulation occurred in MSTN-B and MSTN-2. Mean expression level of MSTN in brain tissue was 2.61 times higher in controls than in MSTN-B (p=7.32e-4).

Significant differences existed between overall expression levels of MSTN in gonad tissue between treatments (p=0.030) (Figure 8). Downregulation occurred in MSTN-A and MSTN-B, while upregulation occurred in MSTN-2. Mean expression level of MSTN in brain tissue was 8.18 times higher in MSTN-2 than in MSTN-A (p=0.028).

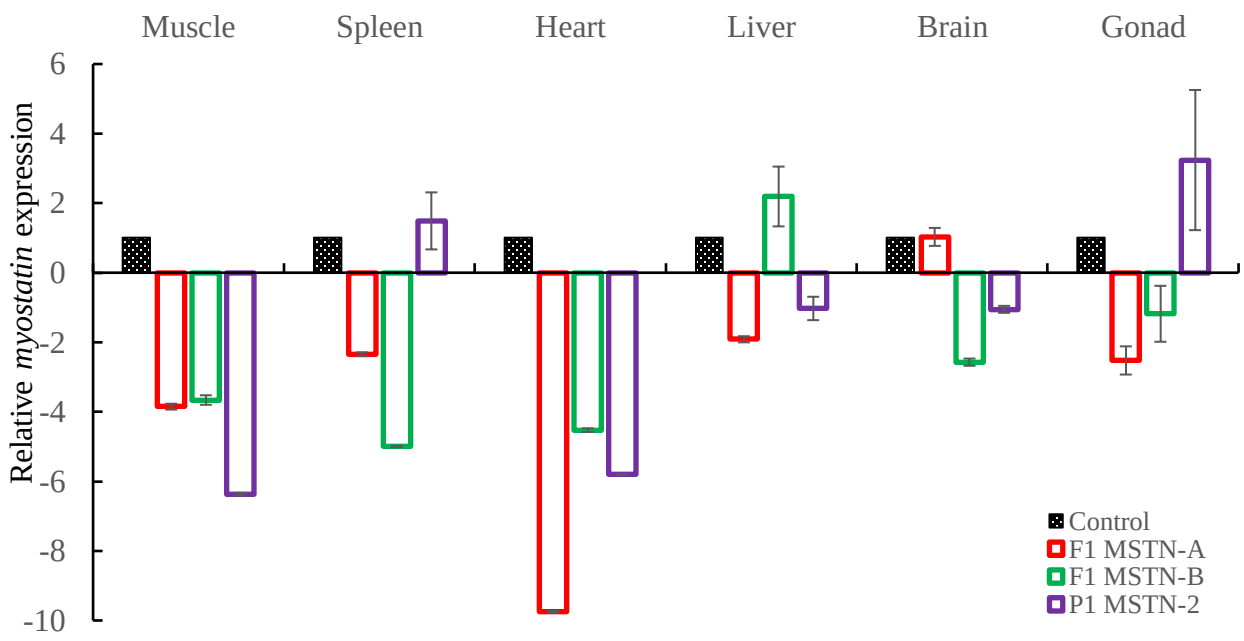


Figure 8. Relative expression of myostatin (MSTN) in various tissues among two F1 heterozygous MSTN mutants from two families (MSTN-A and MSTN-B), one P1 homozygous/bi-allelic MSTN mutant (MSTN-2) and control channel catfish, *Ictalurus punctatus*. Bars below the 0 line indicate reduced MSTN RNA expression from the control while bars above the 0 line indicate increased expression from the control. Error bars indicate standard deviation (N=3 measurements per sample. The fish were 9-months post hatch.

3.5. Enteric Septicemia of Catfish (ESC) Challenge

All families reached 100% mortality within 144-hours after inoculation with *Edwardsiella ictaluri.* Mortality did not begin until 60-hours after inoculation for any family (Figure 9). MSTN-A, CNTRL-A and CNTRL-B all surpassed 50% mortality at 56-hours after inoculation, while MSTN-B reached 50% mortality at 96-hours. Control family 1 (CNTRL-B) was the fastest to reach 100% mortality, reaching it at 108-hours while MSTN-B consistently had the slowest mortality rate throughout the entire challenge, reaching 100% mortality at 144-hours. There was a significant difference in mean hours to death (MHD) post-challenge between families ($p=0.032$). MSTN-B had significantly higher MHD compared to CNTRL-B ($p=0.030$). There were no significant differences in cumulative percent mortality (CPM) between families at any time point ($p>0.05$) (Table 8). The largest difference in CPM between families was at 72 hours ($p=0.204$). Additionally, when both MSTN families were combined and both CNTRL families were combined, there was no significant differences in CPM between genotypes at any time point ($p>0.05$) (Table 8). The AUMPC indices over time are illustrated in Figure 10. There was no significant difference in the total AUMPC between different families ($p=0.241$). The p-values of comparison of AUMPC at each time point after first mortality are 0.633, 0.383, 0.282, 0.334, 0.223, 0.306, 0.375, 0.441 at 60, 72, 84, 96, 108, 120, 132, 144-hours post challenge respectively. Thus, no significant difference was found among the AUMPC indices at any time point ($P>0.05$).

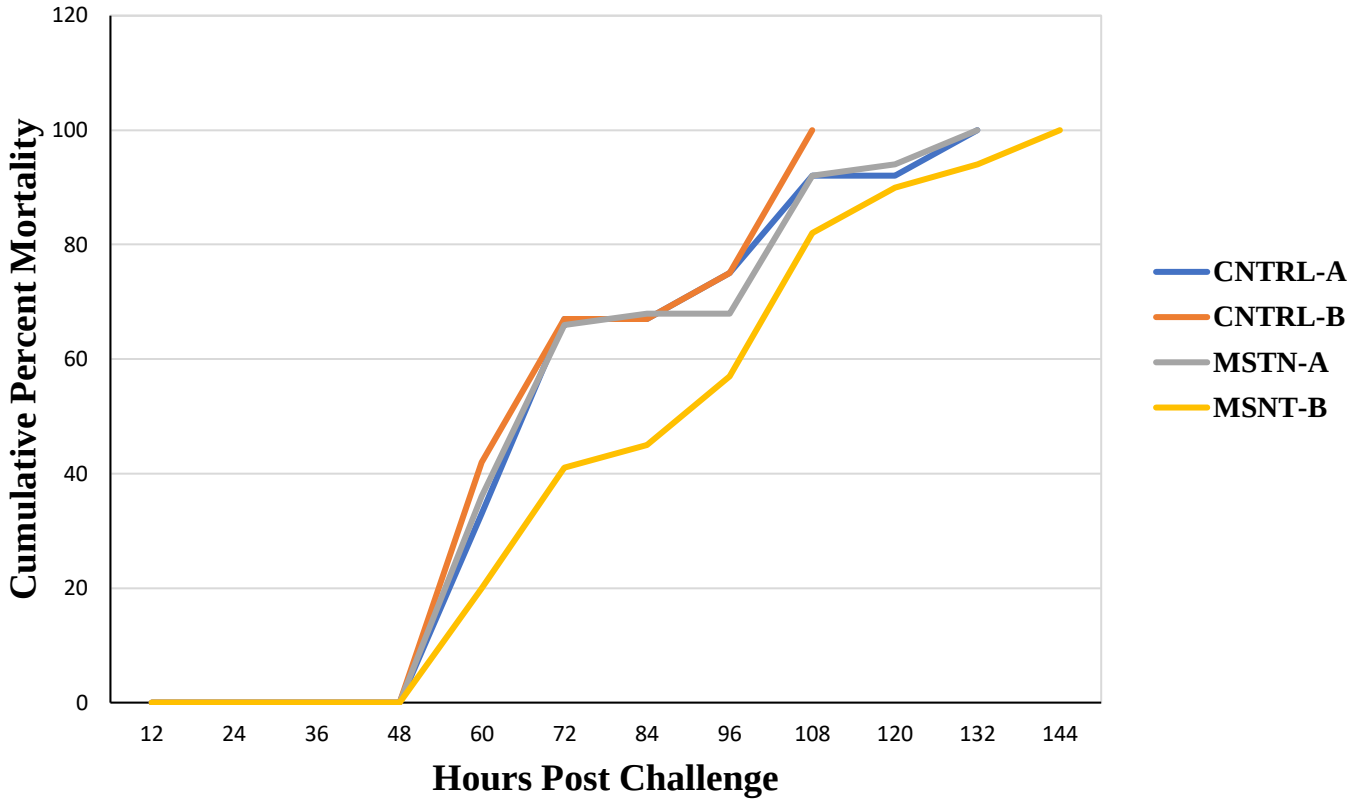


Figure 9. A total of 104 F1 heterozygous myostatin (MSTN) mutant channel catfish, *Ictalurus punctatus*, in two families (MSTN-A and MSTN-B) and 24 control channel catfish in two families (CNTRL-A and CNTRL-B) were challenged with *Edwardsiellla ictaluri*, causative agent for enteric septicemia of catfish (ESC). Fish were naïve and 7-months old, averaging 9.25± 2.55g at the start of the challenge. Fish were checked twice per day for 6 days until each family reached 100% mortality. The lines in the figure each represent the total mortality at each time point for each treatment.

Table 8. Mean cumulative percent mortality (CPM) between F1 myostatin (MSTN) mutant channel catfish, *Ictalurus punctatus,* and wild-type (CNTRL) channel catfish challenged with *Edwardsiellla ictaluri*, causative agent for enteric septicemia of catfish (ESC). A total of 104 F1 heterozygous MSTN mutant channel catfish in two families (MSTN-A and MSTN-B) and 24 control channel catfish in two families (CNTRL-A and CNTRL-B) were challenged and cumulative percent mortality data was analyzed between the combined MSTN families and CNTRL families using a two sample t-test (α priori = 0.05). Fish were naïve and 7-months old, averaging 9.25± 2.55g at the start of the challenge. Fish were checked twice per day for 6 days until each family reached 100% mortality. ANOVA was used to determine significant differences in Family CPM. No significant differences were found between families (p>0.05). Paired t-test was used to determine significant differences in treatment CPM.

	Mean Cumulative Percent Mortality (CPM)						
	60 hours	72 hours	84 hours	96 hours	108 hours	120 hours	132 hours
CNTRL-A	33.33% (4/12)	66.67% (8/12)	66.67% (8/12)	75% (8/12)	91.67% (11/12)	91.67% (11/12)	100% (12/12)
CNTRL_B	41.67% (5/15)	66.67% (8/12)	66.67% (8/12)	83.33% (10/12)	100% (12/12)	100% (12/12)	100% (12/12)
MSTN-A	35.84% (19/53)	66.23% (35/53)	66.23% (35/53)	68.08% (36/53)	92.48% (49/53)	98.04% (52/53)	100% (53/53)
MSTN-B	19.61% (10/51)	39.22% (20/51)	45.1% (23/51)	56.86% (29/51)	82.38% (42/51)	90.2% (46/51)	94.12% (48/51)
CNTRL	37.5% (9/24)	66.67% (16/24)	66.67% (16/24)	75% (18/24)	95.83% (23/24)	95.83% (23/24)	100% (24/24)
MSTN	27.79% (29/104)	53.65% (56/104)	55.77% (58/104)	62.50% (65/104)	87.5% (91/104)	92.31% (96/104)	97.12% (101/104)
p-value	0.4266	0.2222	0.3052	0.09745	0.1572	0.7363	0.221

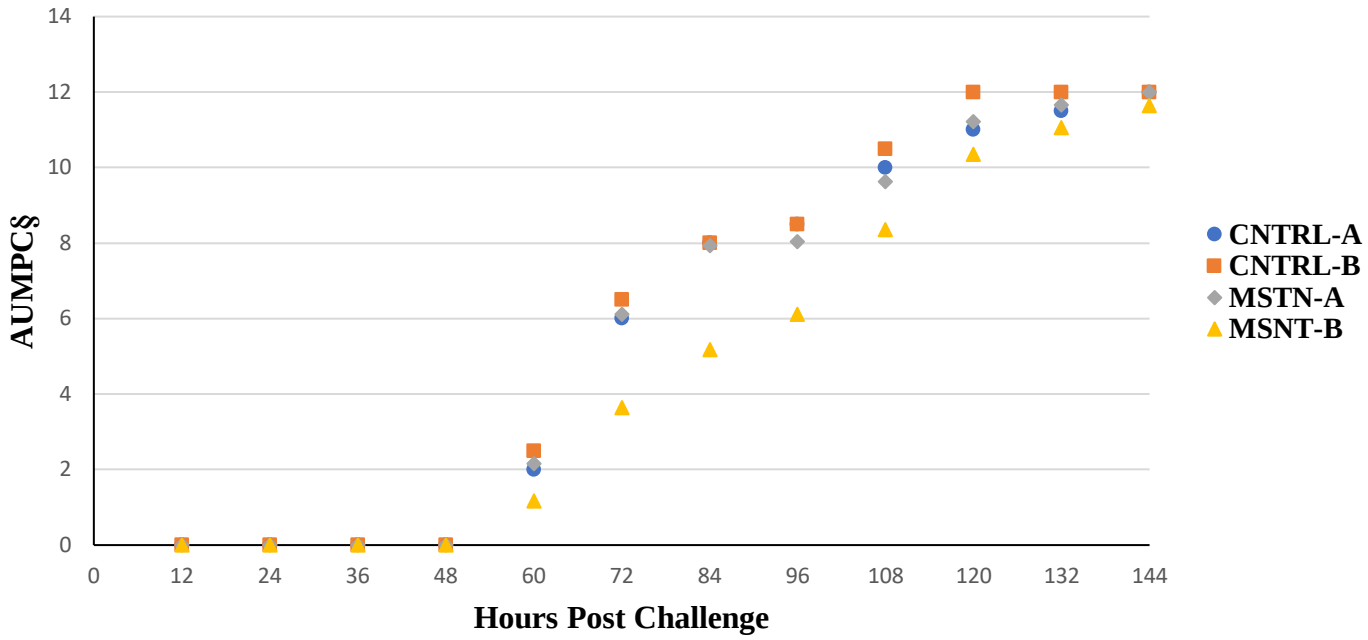


Figure 10. Area under the mortality progress curve (AUMPC) of F1 myostatin (MSTN) mutant channel catfish, *Ictalurus punctatus*, and wild-type (CNTRL) channel catfish challenged with *Edwardsiellla ictaluri*, causative agent for enteric septicemia of catfish (ESC). A total of 104 F1 heterozygous MSTN mutant channel catfish in two families (MSTN-A and MSTN-B) and 24 control channel catfish in two families (CNTRL-A and CNTRL-B) were challenged. Fish were naïve and 7-months old, averaging 9.25± 2.55g at the start of the challenge. Fish were checked twice per day for 6 days until each family reached 100% mortality. No significant differences in the total AUMPC were found between different families (p=0.241). The p-values of comparison of AUMPC at each time point after first mortality are 0.633, 0.383, 0.282, 0.334, 0.223, 0.306, 0.375, 0.441 at 60, 72, 84, 96, 108, 120, 132, 144-hours post challenge respectively.

4. Discussion

In the current study, the effects of microinjection of three sgRNAs targeting exon 1 of the channel catfish myostatin gene in conjunction with Cas9 protein on mutation rate, expression level, growth and disease resistance were investigated. Efficient mutagenesis was achieved as demonstrated by PCR, Surveyor assay and DNA sequencing. The highest mutation rate of 66% was achieved by MSTN-2 gRNA. However, the highest rate of homozygosity/bi-allelism, 84%, was achieved by combining all three gRNAs (MSTN-Mix), indicating that combining multiple successful gRNAs can better cleave both DNA strands. An F1 generation was successfully produced. P1 and F1 mutants both showed enhanced growth, 10-400%. MSTN gene editing resulted in decreased MSTN gene expression. The resistance of the mutants to the bacterial pathogen, *E. ictaluri*, was slightly increased in one MSTN family.

A total of 209 fish survived microinjection over 3 years with an average mutation rate of 58%. Mutation rate showed high variability between years with the lowest rate of 31% in 2017 and highest rate of 100% in 2018. These results are more variable than a previous study where mutation rates of 88-100% were achieved using CRISPR/Cas9 to target MSTN in channel catfish (Khalil et al., 2017). Mutation rates of 60-100 percent using CRISPR/Cas9 are typical for channel catfish for various genes studied at our facility (Qin 2015; Khalil et al., 2017; Elaswad et al., 2018; Qin, 2019). Mutation rates in other teleosts using CRISPR/Cas9 vary considerably between studies, fluctuating between from 2% to 100% (Jao et al., 2013; Chakrapani et al., 2016; Liu et al., 2018). The variability in this study was likely due to a combination of variability of egg quality, period during the spawning season, timing after ovulation and/or human error. Future experiments should test family and strain effects on mutation rate.

MSTN F1 mutants were generated by individually mating two pairs of control females with MSTN mutant males. The offspring of both families inherited the mutation at a rate of 88%, indicating a very low level of mosaicism in the parent generation. As the dam was the control, all mutant offspring were heterozygous. Germline transmission rates range considerably among other studies. Varshney et al. (2015) attained a 53% mutation rate from 162 loci targeting 83 genes in zebrafish and had an average germline transmission rate of 28%. Similar to the current study, mutated founder individuals were mated with wild type fish. Zebrafish progeny generated from mating founders with mutations in the C9t2 and C9t3 genes had germline transmission rates of 43% and 20% respectively (Hruscha et al., 2013). Due to mosaicism, germline transmission of CRISPR/Cas9 induced mutations are not necessarily inherited with Mendelian distributions.

Growth was generally higher in MSTN mutants when compared to controls at all life stages and in both pond and tank environments. At the small fingerling stage (10-20g), P1 MSTN mutants were 30% larger than controls on average. At the stocker stage (100-200g), P1 MSTN mutants were 88% larger than controls on average and P1 MSTN mutants reached market size 27% faster than controls. Heterozygous F1 mutants were 218% larger than controls on average at 100g. These increases in body weight are considerably higher than those in previous studies of teleost fish including zebrafish, medaka, red sea bream, spotted halibut, common carp and olive flounder, where MSTN mutants were 51% larger on average than wild-type and ranged from an 11% decrease to 88% increase in body weight (Kim et al., 2016; Kim et al., 2019; Zong et al., 2016; Wang et al., 2018; Chiang et al., 2016; Khalil et al., 2017; Yeh et al., 2017; Gao et al., 2016; Chisada et al., 2011; Kishimoto et al., 2018; Li et al., 2012).

Growth improvement through MSTN knockout in the current study was superior to typical results from selection, crossbreeding and hybridization in channel catfish. Selection typically yields increases of 7-14% per generation (Gjedrem and Thodesen, 2005; Dunham, 2011). Dunham and Smitherman (1983) found that six of nine (67%) channel catfish crossbreeds showed heterosis for body weight, averaging 10.3 percent above both parents, although 50% improvement is possible. Interspecific hybridization can yield substantial increases in body weight, such as in the case of hybrid catfish, which may grow 20% up to 100% faster than either parent depending upon the parent (Dunham et al., 1990; Dunham, 2011). Transgenesis has the potential for much larger gains in body weight than those achieved in this study. By incorporating the Chinook salmon, *Oncorhynchus tshawytscha*, growth hormone into an Atlantic salmon, *Salmo salar*, growth rate was increased by 400-600 percent, reducing the grow-out period from three years to 18 months (Elliot, 1998; Entis, 1998). However, growth hormone channel catfish grow 60-80% faster than controls (Abass et al., 2020; Abass et al., 2021). While transgenics can reduce grow-out time and cost, there are ethical and environmental concerns with introducing a foreign gene into an organism (Muir and Howard, 1999). Crispr/Cas9 gene knockout, while artificial in methodology produces an end product that could intrinsically come about through natural mutations.

Natural MSTN mutants are already used in terrestrial agriculture as Belgian Blue and Piedmontese cattle breeds are frequently utilized. However, mutations in fish result in larger phenotypic gains than terrestrial animals, as their mutants average 24% larger than wild-type (Kim et al., 2016; Lv et al., 2016; Crispo et al., 2015; McPherron et al., 1997; Wang et al., 2017). The larger gains in teleost fish are likely explained by the larger proportion of muscle to body weight

in fish compared to terrestrial animals (Tlusty et al., 2018) or the indeterminate growth of fish. This faster growth to harvest makes MSTN mutants a potentially and extremely valuable genotype for commercial aquaculture. Future research should evaluate the difference in growth between homozygous and heterozygous F1 MSTN mutants.

The growth of individual MSTN mutants and different MSTN families varied. This is likely due to differences in knockout patterns and subsequent expression levels of MSTN, differences in genetic background coupled with epistatic interaction with other loci or differences in off-target mutations. The variability can be advantageous as combining gene editing with selection for the largest individuals might result in maximum genetic enhancement.

No genotype x environment interactions or age effects were observed. Variation in growth correlated more closely with year class than environment or age. However, only one generation of MSTN mutants reached sexual maturity, and therefore age effects may not be apparent at this time. Normal fish growth slows down after sexual maturity and future studies should evaluate whether MSTN plays a stronger role as the fish continue to grow.

MSTN mutants had reduced overall expression levels of MSTN when compared to controls. Mean expression level of MSTN was 2.90 times higher in controls than in combined MSTN F1 progeny. No one treatment had universally greater reduction in expression across all tissues, and no significant differences existed in expression levels of MSTN between tissues of wild-type fish, indicating MSTN expression is complex and dynamic. Expression was most reduced in muscle, spleen and heart. In muscle, spleen and heart, MSTN expression was reduced

5-fold, 3-fold and 7-fold, respectively. Previous studies indicate that MSTN is predominantly expressed in the brain, muscle and heart tissue (Du et al., 2014; Helterline et al., 2007). Therefore, the greatest reduction in MSTN expression can be expected from the tissues with highest normal expression levels. Due to the importance of optimal brain and heart function for survival, the gross characteristics should be evaluated in future experiments to determine if MSTN mutants have deformities in any organs. However, the MSTN mutants in this study appear normal and healthy, which is further supported and indicated by their rapid growth.

Along with the phenotypic differences and RNA expression levels, DNA sequencing confirmed large mutations in the MSTN gene leading to truncation of the MSTN protein. Deletions were found in each target site, with the MSTN-2 gRNA causing a 2184bp deletion that knocked out nearly the entire MSTN gene. As only the largest mutants from each treatment were sequenced, more mutants should be sequenced to determine whether such large deletions are common. Future experiments should evaluate whether the type of mutation and MSTN expression levels influence growth.

When challenged for resistance to enteric septicemia of catfish (ESC), MSTN mutants performed equally or better than controls. MSTN-A, CNTRL-A and CNTRL-B all surpassed 50% mortality 56-hours after inoculation, while MSTN-B reached 50% mortality at 96-hours. One of two families, MSTN-B, had higher mean hours to death (MHD) compared to CNTRL-B. Thus, there appears to be a slight increase in the bacterial disease resistance of some MSTN genotypes, although more extensive research is needed. This contradicts results by Chiang et al. (2016), in which MSTN mutant medaka challenged with red spotted grouper nervous necrosis virus

(RGNNV) had seemingly compromised immune systems, as indicated by reduced expression levels of interferon-stimulated genes and elevated virus copy numbers, when compared to wild-type medaka. A trade-off between growth and immunity is seen in a variety of animals (Henryon et al., 2002). Additionally, mice with chronic kidney disease and obesity show increased levels of MSTN expression, indicating the gene may play an important immune role (Rahimov and Kunkel, 2013; Zhang et al., 2011).

The non-congruence of the channel catfish with the medaka disease resistance results might be related to channel catfish being unique, having only one copy of the MSTN gene, and channel catfish taking a different evolutionary path. Within diploid teleosts myostatin-b retains its role in muscle development, while myostatin-a relates to immune function (Wang et al., 2018). Channel catfish MSTN is homologous to *mstn-b* in other diploid teleosts, and therefore may not play a role in immune function (Karim et al., 2017; Kocabas et al., 2002; Gregory et al., 2004). More research must be carried out on other diseases and species to determine the role of channel catfish MSTN on immune function, and how it relates to other teleosts.

The improved growth and potentially enhanced disease resistance indicate that the use of gene edited MSTN channel catfish could be beneficial for commercial farms. Catfish farming and production in the United States peaked in 2003, dramatically declined from 2007-2012 and has been gradually increasing since that time (Hanson and Sites, 2015; Torrans and Ott, 2018; FAO, 2020). Gene editing presents a valuable tool to increase profitability, sustainability and industry growth. There are, however, a number of ethical, logistical and regulatory hurdles for the MSTN mutant channel catfish to become applied commercially in the United States, as FDA currently

regulates gene edited animals. The improvement of gene editing technologies, greater understanding of its effects and the commercial success of genetically improved organisms, including Aquabounty's AquaAdvantage salmon make this technology a viable option in the near future. By combining MSTN gene editing with other genetic techniques, such as selection, crossbreeding and hybridization, it is likely possible to achieve even greater growth results, shorten the grow-out period, and select for multiple traits. With an increasing human population and declining natural resources, all solutions should be evaluated to determine the most efficient and sustainable methods of food production.

References

Abass, N. Y., D. Drescher, N. Backenstose, Z. Ye, K. Vo, R. Odin, G. Qin, S. Dong and R. A. Dunham. 2020. Genotype-environment interactions for survival and growth rate at varying levels of sodium chloride for growth hormone transgenic channel catfish (*Ictalurus punctatus*), channel catfish, and albino channel catfish. *Aquaculture, 521*, 735084.

Abass, N. Y., B. Su, D. A. Perera, Z. Qin, H. Li, A. Alsaqufi, A. Elaswad, Z. Ye, S. Dong, R. A. Dunham. 2021.Effects of family and promoter on growth performance of ccGH cDNA transgenic channel catfish, *Ictalurus punctatus*, grown in a trough culture system. *Aquaculture, 536*, 736468.

Chakrapani, V., Patra, S. K., Panda, R. P., Rasal, K. D., Jayasankar, P., & Barman, H. K. (2016). Establishing targeted carp TLR22 gene disruption via homologous recombination using CRISPR/Cas9. *Developmental & Comparative Immunology*, *61*, 242-247.

Chen, Y. T., Lin, C. F., Chen, Y. M., Lo, C. E., Chen, W. E., & Chen, T. Y. (2017). Viral infection upregulates myostatin promoter activity in orange-spotted grouper (*Epinephelus coioides*). *PloS One*, *12*(10), e0186506.

Chiang, Y. A., Kinoshita, M., Maekawa, S., Kulkarni, A., Lo, C. F., Yoshiura, Y., Wang, H. C., & Aoki, T. (2016). TALENs-mediated gene disruption of myostatin produces a larger phenotype of medaka with an apparently compromised immune system. *Fish & Shellfish Immunology*, *48*, 212-220.

Chisada, S. I., Okamoto, H., Taniguchi, Y., Kimori, Y., Toyoda, A., Sakaki, Y., Takeda, S., & Yoshiura, Y. (2011). Myostatin-deficient medaka exhibit a double-muscling phenotype with hyperplasia and hypertrophy, which occur sequentially during post-hatch development. *Developmental Biology*, *359*(1), 82-94.

Clop, A., Marcq, F., Takeda, H., Pirottin, D., Tordoir, X., Bibé, B., Bouix, J., Caiment, F., Elsen, J. M., Eychenne, F., Larzul, C., Laville, E., Meish, F., Milenkovic, D., Tobin, J., Charlier, C., & Georges, M. (2006). A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nature Genetics*, *38*(7), 813-818.

Cuevas-Uribe, R., & Tiersch, T. R. (2011). Estimation of fish sperm concentration by use of spectrophotometry. Cryopreservation in Aquatic Species, 2nd edition. World Aquaculture Society, Baton Rouge, LA, 162-200.

DeLuna, A., Vetsigian, K., Shoresh, N., Hegreness, M., Colón-González, M., Chao, S., & Kishony, R. (2008). Exposing the fitness contribution of duplicated genes. *Nature Genetics*, *40*(5), 676.

DeLuna, A., Springer, M., Kirschner, M. W., & Kishony, R. (2010). Need-based up-regulation of protein levels in response to deletion of their duplicate genes. *PLoS Biol*, *8*(3), e1000347.

Du, F., Xu, G., Nie, Z., Xu, P., & Gu, R. (2014). Molecular characterization and differential expression of the myostatin gene in *Coilia nasus*. *Gene*, *543*(1), 153-160.

Dunham, R. A., & Smitherman, R. O. (1983b). Crossbreeding channel catfish for improvement of body weight in earthen ponds. *Growth*, *47*(1), 97.

Dunham, R. A., Smitherman, R. O., & Goodman, R. K. (1987). Comparison of mass selection, crossbreeding, and hybridization for improving growth of channel catfish. *The Progressive Fish-Culturist*, *49*(4), 293-296.

Dunham, R. A., Brummett, R. E., Ella, M. O., & Smitherman, R. O. (1990). Genotype-environment interactions for growth of blue, channel and hybrid catfish in ponds and cages at varying densities. *Aquaculture*, *85*(1-4), 143-151.

Dunham, R.A. (2011). Aquaculture and fisheries biotechnology: genetic approaches. CABI Wallingford, Oxfordshire, UK

Elaswad, A., Khalil, K., Cline, D., Page-McCaw, P., Chen, W., Michel, M., Cone, R., & Dunham, R. (2018). Microinjection of CRISPR/Cas9 protein into channel catfish, *Ictalurus punctatus*, embryos for gene editing. *JoVE (Journal of Visualized Experiments)*, (131), e56275.

Elliot E. (1998) Aquadvantage salmon: A case study in transgenic food. *Animal Biotechnology,* 9:3, 165-170. DOI: 10.1080/10495399809525906

Entis, E. (1998). Aquadvantage salmon: A case study in transgenic food. *Animal Biotechnology,* 9:3, 165-170

FAO (2020). The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome. https://doi.org/10.4060/ca9229en

Gabillard, J. C., Biga, P. R., Rescan, P. Y., & Seiliez, I. (2013). Revisiting the paradigm of myostatin in vertebrates: insights from fishes. *General and Comparative Endocrinology*, *194*, 45-54.

Gao, Y., Dai, Z., Shi, C., Zhai, G., Jin, X., He, J., Lou, Q., & Yin, Z. (2016). Depletion of myostatin b promotes somatic growth and lipid metabolism in zebrafish. *Frontiers in Endocrinology*, *7*, 88.

Gjedrem, T., & Thodesen, J. (2005). Selection and Breeding Programs in Aquaculture (pp. 89-111). Springer, Dordrecht.

Gregory, D. J., Waldbieser, G. C., & Bosworth, B. G. (2004). Cloning and characterization of myogenic regulatory genes in three Ictalurid species. *Animal Genetics*, *35*(6), 425-430.

Guo, T., Jou, W., Chanturiya, T., Portas, J., Gavrilova, O., & McPherron, A. C. (2009). Myostatin inhibition in muscle, but not adipose tissue, decreases fat mass and improves insulin sensitivity. *PloS One*, *4*(3), e4937.

Guo, R., Wan, Y., Xu, D., Cui, L., Deng, M., Zhang, G., Jia, R., Zhou, W., Wang, Z., Deng, K., Huang, M., Wang, F., & Zhang, Y. (2016). Generation and evaluation of Myostatin knock-out rabbits and goats using CRISPR/Cas9 system. *Scientific Reports*, *6*(1), 1-10.

Hanson, T. & Sites, D. (2015). 2014 U.S. Catfish Database. MSU AEC Information Report. Retrieved from http://www.agecon.msstate.edu/whatwedo/budgets/docs/catfish2014.pdf

Helterline, D. L., Garikipati, D., Stenkamp, D. L., & Rodgers, B. D. (2007). Embryonic and tissue-specific regulation of myostatin-1 and-2 gene expression in zebrafish. *General and Comparative Endocrinology*, *151*(1), 90-97.

Henryon, M., Jokumsen, A., Berg, P., Lund, I., Pedersen, P. B., Olesen, N. J., & Slierendrecht, W. J. (2002). Genetic variation for growth rate, feed conversion efficiency, and disease resistance exists within a farmed population of rainbow trout. *Aquaculture*, *209*(1-4), 59-76.

Hruscha, A., Krawitz, P., Rechenberg, A., Heinrich, V., Hecht, J., Haass, C., & Schmid, B. (2013). Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. *Development*, *140*(24), 4982-4987.

Jao, L. E., Wente, S. R., & Chen, W. (2013). Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proceedings of the National Academy of Sciences*, *110*(34), 13904-13909.

Jeger, M. J., & Viljanen-Rollinson, S. L. H. (2001). The use of the area under the disease-progress curve (AUDPC) to assess quantitative disease resistance in crop cultivars. *Theoretical and Applied Genetics*, *102*(1), 32-40.

Khalil, K., Elayat, M., Khalifa, E., Daghash, S., Elaswad, A., Miller, M., Abdelrahman, H., Ye, Z., Odin, R., Drescher, D., Vo, K., Gosh, K., Bugg, W., Ribinson, D., & Dunham, R. (2017). Generation of myostatin gene edited channel catfish (*Ictalurus punctatus*) via zygote injection of CRISPR/Cas9 system. *Scientific Reports*, *7*(1), 1-12.

Kim, J., Cho, J. Y., Kim, J. W., Kim, H. C., Noh, J. K., Kim, Y. O., Hwang, H. K., Kim, W. J., Yeo, A. Y., Park, J. Y., & Kong, H. J. (2019). CRISPR/Cas9-mediated myostatin disruption enhances muscle mass in the olive flounder *Paralichthys olivaceus*. *Aquaculture*, *512*, 734336.

Kim, G. D., Lee, J. H., Song, S., Kim, S. W., Han, J. S., Shin, S. P., Park, B. C., & Park, T. S. (2020). Generation of myostatin-knockout chickens mediated by D10A-Cas9 nickase. *The FASEB Journal*, *34*(4), 5688-5696.

Kishimoto, K., Washio, Y., Yoshiura, Y., Toyoda, A., Ueno, T., Fukuyama, H., Kato, K., & Kinoshita, M. (2018). Production of a breed of red sea bream Pagrus major with an increase of skeletal muscle mass and reduced body length by genome editing with CRISPR/Cas9. *Aquaculture*, *495*, 415-427.

Kocabas, A. M., Kucuktas, H., Dunham, R. A., & Liu, Z. (2002). Molecular characterization and differential expression of the myostatin gene in channel catfish (*Ictalurus punctatus*). *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, *1575*(1-3), 99-107.

Li, Y., Tian, M., Xie, H., Guo, T., Niu, J., Liu, Y., & Tian, L. (2017). Effects of myostatin inhibitory peptides supplementation on growth performance, body composition, serum biochemical indices, serum and liver immune indices of sea bass (*Lateolabrax japonicas*). *Chinese Journal of Animal Nutrition*, *29*(2), 519-528.

Li, H., Fan, J., Liu, S., Yang, Q., Mu, G., & He, C. (2012). Characterization of a myostatin gene (MSTN1) from spotted halibut (*Verasper variegatus*) and association between its promoter polymorphism and individual growth performance. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *161*(4), 315-322.

Liu, Q., Yuan, Y., Zhu, F., Hong, Y., & Ge, R. (2018). Efficient genome editing using CRISPR/Cas9 ribonucleoprotein approach in cultured Medaka fish cells. *Biology Open*, *7*(8).

Lv, X. Y., Sun, W., Su, R., Li, D., Wang, Q. Z., Musa, H. H., Chen, L., Zhang, X. Y., & Wu, W. Z. (2015). Correlation between sheep YAP1 temporal and spatial expression trends and MSTN and MyoG gene expression. *Genetics and Molecular Research*, *14*(2), 3244-3256.

Lv, Q., Yuan, L., Deng, J., Chen, M., Wang, Y., Zeng, J., Li, Z., & Lai, L. (2016). Efficient generation of myostatin gene mutated rabbit by CRISPR/Cas9. *Scientific Reports*, *6*, 25029.

Mosher, D. S., Quignon, P., Bustamante, C. D., Sutter, N. B., Mellersh, C. S., Parker, H. G., & Ostrander, E. A. (2007). A mutation in the myostatin gene increases muscle mass and enhances racing performance in heterozygote dogs. *PLoS Genetics*, *3*(5), e79.

Muir, W. M., & Howard, R. D. (1999). Possible ecological risks of transgenic organism release when transgenes affect mating success: sexual selection and the Trojan gene hypothesis. *Proceedings of the National Academy of Sciences*, *96*(24), 13853-13856.

Nowak, M. A., Boerlijst, M. C., Cooke, J., & Smith, J. M. (1997). Evolution of genetic redundancy. *Nature*, *388*(6638), 167-171.

Phelps, R. P., Hastey, R., Pendetar, A., Linley, L., Papanikos, N., & Dunham, R. A. (2007). Effects of temperature on the induced spawning of channel catfish and the production of channel× blue catfish hybrid fry. *Aquaculture*, *273*(1), 80-86.

Qin, Z. (2015). Gene editing of luteinizing hormone, follicle-stimulating hormone and gonadotropin-releasing hormone genes to sterilize channel catfish, *Ictalurus punctatus*, using zinc finger nuclease, transcription activator-like effector nuclease and clustered regularly interspaced short palindromic repeats/Cas9 technologies. Doctoral dissertation, Auburn University, AL, USA.

Qin, G. (2019). Gene Editing and Hormone Therapy to Control Reproduction in Channel Catfish, *Ictalurus punctatus*. Doctoral dissertation, Auburn University, AL, USA.

Qin, Z., Li, Y., Su, B., Cheng, Q., Ye, Z., Perera, D. A., Fobes, M., Shang, M., & Dunham, R. A. (2016). Editing of the luteinizing hormone gene to sterilize channel catfish, *Ictalurus punctatus*, using a modified zinc finger nuclease technology with electroporation. *Marine Biotechnology*, *18*(2), 255-263.

Rahimov, F., & Kunkel, L. M. (2013). Cellular and molecular mechanisms underlying muscular dystrophy. *Journal of Cell Biology*, *201*(4), 499-510.

Tlusty, M., Tyedmers, P., Ziegler, F., Jonell, M., Henriksson, P., Newton, R., ... & Cao, L. (2018). Commentary: comparing efficiency in aquatic and terrestrial animal production systems. *Environmental Research Letters*, *13*(12).

Torrans, L., & Ott, B. (2018). Effect of grading fingerling hybrid catfish (♀ channel catfish×♂ blue catfish) on growth, production, feed conversion, and food fish size distribution. *North American Journal of Aquaculture*, *80*(2), 187-192.

Varshney, G. K., Pei, W., LaFave, M. C., Idol, J., Xu, L., Gallardo, V., Carrington, B., Bishop, K., Jones, M. P., Li, M., Harper, U., Huang, S. C., Prakash, A., Chen, W., Sood, R., Ledin, J. & Burgess, S. M. (2015). High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. *Genome Research*, *25*(7), 1030-1042.

Wang, C., Chen, Y. L., Bian, W. P., Xie, S. L., Qi, G. L., Liu, L., Strauss, P. R., Zou, J. X., & Pei, D. S. (2018). Deletion of MSTNa and MSTNb impairs the immune system and affects growth performance in zebrafish. *Fish & Shellfish Immunology*, *72*, 572-580.

Wang, K., Ouyang, H., Xie, Z., Yao, C., Guo, N., Li, M., Jiao, H., & Pang, D. (2015). Efficient generation of myostatin mutations in pigs using the CRISPR/Cas9 system. *Scientific Reports*, *5*, 16623.

Wang, X., Niu, Y., Zhou, J., Zhu, H., Ma, B., Yu, H., Qu, L., & Chen, Y. (2018). CRISPR/Cas9-mediated MSTN disruption and heritable mutagenesis in goats causes increased body mass. *Animal Genetics*, *49*(1), 43-51.

Wu, H. H., Ivkovic, S., Murray, R. C., Jaramillo, S., Lyons, K. M., Johnson, J. E., & Calof, A. L. (2003). Autoregulation of neurogenesis by GDF11. *Neuron*, *37*(2), 197-207.

Xia, Z., Wei, J., Li, Y., Wang, J., Li, W., Wang, K., Hong, X., Zhao, L., Chen, C., Min, J., & Wang, F. (2017). Zebrafish slc30a10 deficiency revealed a novel compensatory mechanism of Atp2c1 in maintaining manganese homeostasis. *PLoS Genetics*, *13*(7), e1006892.

Ye, X., Brown, S. R., Nones, K., Coutinho, L. L., Dekkers, J. C., & Lamont, S. J. (2007). Associations of myostatin gene polymorphisms with performance and mortality traits in broiler chickens. *Genetics Selection Evolution*, *39*(1), 1-17.

Zhang, L., Rajan, V., Lin, E., Hu, Z., Han, H. Q., Zhou, X., Song, Y., Min, H., Wang, X., Du, J., & Mitch, W. E. (2011). Pharmacological inhibition of myostatin suppresses systemic inflammation and muscle atrophy in mice with chronic kidney disease. *The FASEB Journal*, *25*(5), 1653-1663.

Zhang, C., Liu, Y., Xu, D., Wen, Q., Li, X., Zhang, W., & Yang, L. (2012). Polymorphisms of myostatin gene (MSTN) in four goat breeds and their effects on Boer goat growth performance. *Molecular Biology Reports*, *39*(3), 3081-3087.

Zhong, Z., Niu, P., Wang, M., Huang, G., Xu, S., Sun, Y., Xu, X., Hou, Y., Sun, X., Yan, Y., & Wang, H. (2016). Targeted disruption of sp7 and myostatin with CRISPR-Cas9 results in severe bone defects and more muscular cells in common carp. *Scientific Reports*, *6*, 22953.

Chapter 3: Growth and Survival of Melanocortin-4 Receptor Gene Edited Channel Catfish, *Ictalurus punctatus*

Abstract

The effects of CRISPR/Cas9 knockout of the melanocortin-4 recepter (MC4R) gene in channel catfish, *Ictalurus punctatus*, was investigated. Three sgRNAs targeting exon 1 of the channel catfish MC4R gene in conjunction with Cas9 protein were microinjected in embryos and mutation rate, expression, inheritance, survival and growth were studied. Efficient mutagenesis was achieved as demonstrated by PCR, Surveyor assay and DNA sequencing. SgRNA targeting exon one of the MC4R gene resulted in an overall mutation rate of 33% and 33% homozygosity/bi-allelism in 2017. MC4R mutants appeared to be sterile and initial attempts at spawning MC4R mutants in 2016 and 2017 using 100 µg/kg Luteinizing Hormone Releasing Hormone analog (LHRHa) were unsuccessful. In 2018 and 2019, MC4R F1 mutants were generated by injecting male and female P1 MC4R broodstock with 40µg/kg LHRHa and 1600 international unit's Human chorionic gonadotropin (HCG). Approximately 71% of progeny inherited the mutation. Growth was generally higher in MC4R mutants when compared to controls (CNTRL) at all life stages and in pond and tank environments. At the stocker stage (~50g), P1 MC4R mutants observed body weight was 19% larger than controls, but not significantly (p=0.522). Observed body weights at the lower end of market size (500g) and at market size for P1 MC4R mutants were 48% larger than controls, but not significantly (p=0.151) and 14% larger than controls, but not significantly (p=0.314), respectively. There were no significant differences in body weight between MC4R F1 progeny and controls generated in 2018, although there was a positive relationship between zygosity and growth, with F1 homozygous/bi-allelic mutants reaching market size 30% faster than

F1 heterozygotes in earthen ponds (p=0.022). At the stocker stage (~50g) MC4RxMC4R mutants generated in 2019 and grown in earthen ponds were 40% larger than the mean of both CNTRLxCNTRL families (p=0.005) and 54% larger than F1 MC4R x CNTRL mutants (p=0.001) as the mutation appears to be recessive. With a high mutation rate and inheritance of the mutation as well as improved growth, the use of gene edited MC4R channel catfish appears to be beneficial for application on commercial farms.

Keywords: Channel catfish, *Ictalurus punctatus*, melanocortin-4 receptor, MC4R, growth, gene editing, CRISPR/Cas9

1. Introduction

The melanocortin-4 receptor (MC4R) is a G protein-coupled receptor within the five-membered melanocortin receptor family (MC1R-MC5R) (Liu et al., 2019). This family of receptors regulate many functions, with MC4R primarily controlling energy homeostasis (Liu et al., 2019). MC4R is expressed in the hypothalamus and is activated by α-melanocyte stimulating hormone (α-MSH), a neuropeptide derived from proopiomelanocortin (POMC) (Liu et al., 2019) and antagonized by agouti-related peptide (AGRP), responsible for increasing appetite and decreasing metabolism and energy expenditure (Liu et al., 2019). MC4R is located upstream of kisspeptin and downstream of leptin and ghrelin, making it an essential metabolic component (Liu et al., 2019). Channel catfish MC4R gene is a two-exon gene with a transcript of 5,258 bp located on chromosome 20 (GenBank Accession No. LBML01001141.1).

In mammals, MC4R is predominantly expressed in the central nervous system (Liu et al., 2019). MC4R plays a key role in feeding inhibition with reduced expression leading to increased appetite (Fan et al., 1997). Homozygous MC4R deficient mice, *Mus musculus*, showed hyperphagic obesity and hyperinsulinemia, while heterozygous mice exhibited an intermediate phenotype (Vaisse et al., 1998). In humans, MC4R mutations are the leading genetic cause of obesity with some studies reporting up to 4% of early-onset obesity cases being caused by a missense of nonsense mutation in the gene (Carroll et al., 2005). Due to its effect on body weight and energy homeostasis, MC4R is a potentially valuable gene for improving growth and feed conversion traits in livestock.

In fish, MC4R plays a role in many physiological processes including appetite and energy expenditure (Cerda-Reverter et al., 2003; Schjolden et al., 2009). In zebrafish, *Danio* rerio, MC4R is largely controlled by MRAP2, which is found in two forms. Larval zebrafish produce MRAP2a, which down-regulates MC4R, thereby increasing appetite, and adults produce MRAP2b, which up-regulates MC4R (Liu et al., 2019). Similarly, CRISPR/Cas9 MC4R knockout zebrafish showed no phenotypic differences from wild-type individuals before 2.5 months post fertilization, while adults displayed increased food consumption, increased growth and higher body fat percentage compared to wild-type individuals (Fei et al., 2017). This compliments the natural growth rate of most fish, with larval fish exhibiting fast growth rate, which gradually slows down throughout its life and typically asymptotes after sexually maturity. Goldfish, *Carassius auratus*, and rainbow trout, *Oncorhynchus mykiss*, that were administered the MC4R agonist MTII showed feed inhibition, while those injected with MC4R antagonist, HSO24 had increased feed intake and subsequent growth increases (Cerda-Reverter et al., 2003; Schjolden et al., 2009). Transgenic zebrafish overexpressing the MC4R antagonist agouti-related protein (AgRP) exhibited obesity, increased linear growth and adipocyte hypertrophy (Song and Cone, 2007).

Much of the research to date focused on SNPs and other natural mutations in the MC4R gene. In a study of Holland's carp, *Spinibarbus hollandi*, SNPs in the MC4R promoter region were associated with reduced expression levels of the gene and subsequently exhibited extremely high growth performance (Yang et al., 2018). In a study on F2 Mangalitsa x Pietrain pigs, *Sus scrofa domesticus*, heterozygous animals containing one of two mutations in the MC4R gene consumed 118g more food and gained 32g more per day than homozygous wild-types (Meidtner et al., 2006).

The ease and precision of CRISPR/Cas9 and ability to generate large mutations that could potentially lead to larger phenotypic gains make the technology ideal for commercial use.

Previous studies show high mutation rates in MC4R gene edited fish. In a study by Xie et al. (2016), the mloxP gene was inserted into the zebrafish genome at the MC4R locus. Using a novel method in which oocytes are injected in advance and incubated in storage medium before fertilization, they were able to achieve a mutation rate of 94.4% in the MC4R locus in P1 individuals. Additionally, the germline transmission for MC4R mutation was 96.7%. Kawahara et al. (2015) achieved a 95% mutation rate in the MC4R locus of medaka, *Oryzias latipes*, using TALENs (Kawahara et al., 2015).

MC4R is a useful candidate not only for its role in body weight but also in fat production. In both brown and brindle cattle, *Bos taurus*, the C1069G SNP of the MC4R gene was associated with increased marbling (Lee et al., 2013). However, there was no association between the same SNP and marbling in a third breed, or any effect on carcass weight in any of the three breeds, indicating that certain mutations may only have commercial value in specific populations and species (Lee et al., 2013). If MC4R mutations cause a similar increase in fat production in fish, particularly in healthy omega-3 fatty acids, it could greatly increase economic value for its health and flavor qualities.

A third potentially useful trait of MC4R is its role in reproduction. Polymorphisms in swordtail and platyfish, *Xiphophorus spp.*, males caused a nearly doubling of body weight as well as a delayed onset of sexual maturity and significant charge in mating behavior (Lampert et al.,

2010). Large males with this polymorphism have complex mating rituals with females, while small, fast maturing males with normal MC4R genes exhibit sneak mating with females. The role of MC4R in reproduction varies between species and even sex. In mice, deletion of the MC4R gene causes hyperphagia, hyperinsulinemia, obesity and progressive infertility in females (Chen et al., 2017). MC4R knockout female mice had reduced levels of luteinizing hormone (*lh*), leading to irregular ovulation and reproductive ability, while males had no difference in testosterone levels (Chen et al., 2017). Medaka with mutations in the MC4R gene showed greater growth and slower embryonic development but no difference in onset of puberty (Liu et al., 2019). In spotted scat, *Scatophagus argus*, MC4R agonists NDP-MSH and THIQ significantly increased levels of primary reproductive hormones *gnrh* (Gonadotropin releasing hormone), *fshb* (Follicle-stimulating hormone beta subunit) and *lhb* (Luteinizing hormone beta subunit), while MC4R antagonists, SHU9119 Ipsen 5i reduced levels of these three hormones (Jiang et al., 2017). This study indicates that MC4R participates in reproductive regulation in fish by stimulating *gnrh* expression in the hypothalamus and *fshb* and *lhb* in the pituitary (Jiang et al., 2017).

Many fish raised in captivity exhibit reproductive problems as they do not experience the same conditions that they would in the wild during spawning. Females often fail to produce mature oocytes or undergo spawning behavior while males often produce lower quality and quantity of milt (Zohar and Mylonas, 2001). The most common solutions are environmental manipulation and hormone therapy. Reproductive hormones have been regularly used since the 1930's to induce final gamete maturation and spawning (Zohar and Mylonas, 2001). While early methods included ground up pituitaries containing gonadotropins, modern synthetic agonists and analogs, including LHRHa, GnRHa, and LHa exhibit increased potency and efficacy. These methods may overcome

potential hormonal issues associated with MC4R mutations and allow germline transmission of the valuable mutation. By combining gene editing of the MC4R gene with hormone therapy, a farmer could have full reproductive control of their fish. They could therefore prevent unwanted mating, potentially prevent a slowdown in growth often associated with gonadal development and reduce environmental risk of escapement. A farmer could then restore fertility using hormonal therapy when required for spawning.

Successful knockout of MC4R and spawning of mutant broodstock does not guarantee that the mutation will be inherited in their progeny. Hruscha et al. (2013) achieved a 50% mutation rate in zebrafish using CRISPR/Cas9, but only 11% of progeny inherited the mutation. Varshney et al. (2015) attained a 53% mutation rate from 162 loci targeting 83 genes in zebrafish and had an average germline transmission rate of 28%. In this study however, mutated founder individuals were mated with wild-type fish, so that parents heterozygous for the mutation would skew the results towards a lower transmission rate. The study also included the 47% of the founders that did not carry mutations in their breeding program and results, further lowering the reported transmission rate. In a study on MC4R mutant mice and rats, *Rattus norvegicus*, half of the offspring of MC4R mutant rat crossed with wild-type rats tested positive for the mutation, indicating a high efficiency of germline transmission after CRISPR/Cas9 mutagenesis. CRISPR/Cas9 mediated MC4R mutant rats were 43% larger than wild-type individuals and showed increased food intake and larger perirenal fat pads (Li et al., 2013). Still, due to mosaicism, epistasis and other unforeseen effects, germline transmission of CRISPR/Cas9 induced mutations are not necessarily inherited with Mendelian distributions. The rate of transmission is also largely

dependent on the species, target gene, gRNA design, genotype of the parents and administration procedure.

The objectives of this study were to knock out the melanocortin-4 receptor (MC4R) gene in channel catfish, evaluate growth and survival, test inheritance of the mutation in the F1 generation and evaluate how zygosity effects growth rate. To our knowledge this is the first study using CRISPR/Cas9 to induce a mutation in the MC4R gene of a commercial food-fish species.

2. Materials and Methods

All experiments were conducted at the Fish Genetics Research Unit, E. W. Shell Fisheries Research Center, Auburn University, AL. All experimental protocols used in this experiment were approved by the Auburn University Institutional Animal Care and Use Committee (AUIACUC) before the experiment was initiated and followed the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) protocols and guidelines.

2.1. Design and preparation of sgRNA and CRISPR/Cas9 System

Three customized small guide RNAs (sgRNAs) were designed and generated using the Maxiscript T7 PCR-based method. First, four gene-specific oligonucleotides (MC4R-A, MC4R-B, MC4R-C, MC4R-D; Table 9) containing the PAM were designed using the CRISPRscan online tool to target the channel catfish MC4R gene (GenBank Accession No. LBML01001141.1). Exon 1 was targeted in all four sgRNA sites in order to truncate the protein as early as possible to have the largest knockout effect. The Universal Primer (Table 9), containing the sgRNA scaffold, was ordered through Thermo Fisher Scientific. Each of the oligos was reconstituted using DNase/RNase Free water to 10mM. To create template for sgRNA synthesis, the three oligos were used to were synthesize double stranded DNA (dsDNA) by mixing 25µl 2x EconoTaq Plus Master Mix (Lucigen), 12.5µl Universal Primer, and 12.5µl gene-specific oligonucleotide. PCR cycling was carried out with initial denaturing at 95°C for 3 min; 5 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec with a ramp speed of -0.2°C/sec, extension at 72°C for 30 sec; 20 cycles of denaturation at 95°C for 30 sec, extension at 72°C for 20 sec with a ramp speed of -0.2°C/sec; and final extension at 72°C for 10 min.

Table 9. Primers used to amplify partial sequences of channel catfish, *Ictalurus punctatus*, oligonucleotides to target specified regions for CRISPR/Cas9 cleavage and single-stranded oligo donor nucleotide (ssODN). Universal primer was used to bind oligonucleotides to Cas-9 protein. Red letters indicate binding site to target gRNAs. MC4R-A, MC4R-B, MC4R-C, MC4R-A2-sense and MC4R-D-antisense were all used to target various loci in exon 1 of the channel catfish MC4R gene. MSTN-1, MSTN-2 and MSTN-3 were all used to target various loci in exon 1 of the channel catfish MC4R gene. MC4R-ALL-1F and MC4R-ALL-1R were used to amplify DNA segments flanking the target sites for guide RNAs in the catfish MC4R gene. EX1-F and EX1-R were used to amplify DNA segments flanking the target sites for guide RNAs in the catfish MC4R gene. PUC57 Vector was used to amplify a partial sequence of the Evol2 plasmid containing the elongase gene. BsalElvol2_ ssODN1 and BsalElvol2_ ssODN2 were used to flank the cut site associated with the MC4R-A2-sense gRNA to facilitate Homologous Directed Repair (HDR) in the MC4R gene. BsalElvol2_ ssODN3 and BsalElvol2_ ssODN4 were used to flank the cut site associated with the MC4R-D-antisense gRNA to facilitate Homologous Directed Repair (HDR) in the MC4R gene.

Oligo sequence (5' to 3')	Oligo name
AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	Universal Primer
taatacgactcactataGGGATGGCGCTGATCACCAGgttttagagctagaa	MC4R-A
taatacgactcactataGGGAAAGGAACTCGGAGTCgttttagagctagaa	MC4R-B
taatacgactcactataGGGCAGGATGGTGAGCGTCAgttttagagctagaa	MC4R-C
taatacgactcactataGGTGGTGGCGTTCGGTCCGAgttttagagctagaa	MSTN1
taatacgactcactataGGGCGAGGCGCAGTGTTCAGgttttagagctagaa	MSTN2
taatacgactcactataGGGTCGAGCAGCTGCTGCACgttttagagctagaa	MSTN3
GGAGATGGAGGACACGGAAG	MC4R-ALL-1F
GAGACATGAAGCAGACGCAATA	MC4R-ALL-1R
taatacgactcactataGTGATGGCGCTGATCACCAGgttttagagctagaa	MC4R-A2-sense
taatacgactcactataCGGGATGCAGCATGCACACCgttttagagctagaa	MC4R-D-antisense
taatacgactcactataCTTGTCTGTAAGCGGATGCCgttttagagctagaa	PUC57 Vector
TATCGAACGCGACAGAAACGGCTGTGATGGCGCTGATCACGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTG	BsalElvol2_ ssODN1
GCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATCAGCGGCAACCTGACCATCTCTGGAGACGTCGTGAAAAGC	BsalElvol2_ ssODN2
GAGGAGGTCTTGCGGATATGAACGTGTCGGAGCACCACGGGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTG	BsalElvol2_ ssODN3
GCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGATGCAGCATGCACACCGGAACCACAGCCTGGGCGTGCAG	BsalElvol2_ ssODN4
ACTCCTCTCTGAGACCTGAC	EX1-F
AGTTAGGCTATGAAGCAGTAGT	EX1-R
CGAAATCCGTTCCTTTTTACTG	Evol2-F
CTGGCCTGTTCCTCATGTATTT	Evol2-R

The Taq Polymerase was then inactivated using 4.8µl of 0.5M EDTA and incubated at 75°C for 20 min. The PCR product was confirmed using 1% agarose gel. The sgRNA was synthesized using the Maxiscript T7 Kit (Thermo Fisher Scientific), following the manufacture guidelines. A mix of 2µl dH2O, 10µl dsDNA Template (created above), 2µl 10X buffer, 1µl ATP, 1µl CTP, 1µl GTP, 1µl UTP and 2µl Enzyme Mix was incubated at 37°C for 90 min. The solution was cleared of DNA contamination by adding 1µl of Turbo DNase I, vortexing briefly and incubating at 37°C for 15 min. The magnesium ions were chelated by adding 5µl of 0.5M EDTA. The Turbo DNase I was inactivated by heating the solution at 75°C for 10 min. The obtained sgRNAs were purified using Zymo RNA Clean and Concentrator kit (Zymo Research). The sgRNAs were stored in -80°C freezer.

The Cas9 protein was acquired from PNA Bio (3541 Old Conejo Rd, Newbury Park, CA 91320) and reconstituted in dH_20 to a concentration of 1mg/ml. Twenty minutes prior to fertilizing the eggs, four sets of injection solutions were prepared by mixing equal parts of Cas9 protein with each of the sgRNAs individually, and a fourth solution combining all three sgRNA with Cas9 protein (MC4Rmix). The mixture was incubated on ice for 10 minutes before adding phenol red to a total ratio of 1:1:1 of Cas9, sgRNA and Phenol red, respectively. The final concentrations of Cas9 protein and sgRNA were 300-350ng/µl and 150-200ng/µl, respectively.

2.2. Brood stock husbandry, selection and spawning

Brood stock were cultured in 0.04-ha earthen ponds averaging 1-meter in depth. They were fed a 32 percent protein catfish pellet at 1-2% of their body weight five days per week. Dissolved oxygen was maintained above 3 mg/L using a ½ horsepower surface aerator (Air-O-Lator).

The Kansas strain of channel catfish was chosen as broodstock due to their superior growth and fry output when induced by injection of luteinizing hormone releasing hormone analogs (LHRHa). Individuals were chosen based on their health and secondary sexual characteristics. Males with well-developed papilla and large, muscular heads that were wider than the rest of their bodies were chosen. Dark coloring and scarring from territorial fighting were also signs of quality males in reproductive condition. Females with soft, well-rounded abdomen that were wider than their head, and a swollen urogenital opening were chosen. Broodstock were minimally handled and kept in tanks for as short a time as possible to avoid stress.

Males were terminated by a percussive blow to the head followed by pithing. The body cavity was opened carefully with a scalpel, ensuring not to pierce any organs. Testes were removed using tweezers and/or scissors and washed in a weigh boat with 0.9 percent saline using bottled distilled water, removing any blood or tissue. The water was drained, and the testes weighed before macerating the testes using scissors to release sperm. The homogenized testes were then filtered into a 50mL falcon tube using a 100-micron screen. The sperm was then diluted with 0.9 percent saline solution to a maximum of 10mL/gram of testes. Sperm concentration was tested using a Nanodrop 2000 Spectrophotometer (Thermo Scientific) and running a simple linear regression between sperm concentration and absorbance using the equation $y=-2.450+0.185\ln X$ (Adjusted $R^2=0.601$) at 450nm (Cuevas-Uribe & Tiersch, 2011). Motility was analyzed under a 10X light microscope while sperm count was checked under 40X magnification. Ideally, sperm was used immediately, but could be stored at 4°C for one week,

with declining quality or up to two weeks with the addition of gentamycin at a concentration of 1mg/mL sperm solution.

Females were weighed and gently placed in labeled 32mm mesh bags. The spawning bags were then placed in a flow through holding tank so that the fish was fully submerged, ensuring that water quality was ideal with dissolved oxygen levels above 5 mg/L. A 14-gauge implanter was loaded with 100µg/kg body weight of luteinizing hormone releasing hormone analog (LHRHa) implant. The needle was then inserted at a 45-degree angle ventrally adjacent to the pelvic fin and the implant was inserted. The ovulation time was predicted based on the degree-hours according to Phelps et al. (2007). Water temperature ranged from 26-28°C. Females were checked 36 hours after LHRHa injection and every four hours after that until ovulation. Once a female began ovulating, indicated by eggs visible on the bag, she was carefully transferred into a tank with 100 mg/L buffered tricaine methane sulfonate (MS-222) with equal parts sodium bicarbonate until completely anesthetized. Prior to this time, a thin layer of vegetable shortening, Crisco, was used to coat several 20cm diameter metal egg collection pans. The fish was then dipped in fresh water to wash off the MS-222 and gently dried with a towel. A thick layer of vegetable shortening was applied around the urogenital opening, including the pelvic fins to prevent sticking of the eggs during stripping. The female was hand stripped into the greased pan by gently applying pressure on the abdomen from the anterior end of the abdomen posteriorly towards the vent. Eggs would usually flow freely, well rounded and golden in color, with minimal blood. The stripping ensured that the eggs could not contact freshwater, which can activate the eggs. Eggs were then covered with another spawning pan to maintain moisture and transported to the molecular laboratory.

Approximately 200-300 eggs were transferred to a greased pan for fertilization. Approximately 3mL of sperm solution was added to the eggs and mixed gently with fingertips. Fresh water was added to barely cover the eggs in order to activate the sperm and eggs and the water was swirled to form a single layer and prevent sticking. After two minutes, the eggs should be fertilized, and three more cm of water was added to the eggs and the eggs were left to harden for 15 minutes.

While the embryos were hardening, 5-10µl of the Cas9/sgRNA/phenol red mixture was loaded into 1.0mm OD borosilicate glass capillary microinjection needles using a microloader. The tips of the needles were opened by breaking the end with a scalpel. The needle was then inserted into the micropipette holder to its deepest range and tightened, ensuring a tight seal. The compressed air cylinder was opened, and pressure was adjusted to 7000kPa and 0.824 m^3/hr using the pressure regulator. The injection volume was adjusted to 50nl by manipulating the pressure, the length of injection and the needle diameter. Injection volume was measured by injecting a drop of mineral oil on a hemocytometer.

After 15 minutes 100-200 embryos were transferred in a single layer to a greased 100mm petri dish and covered with Holtfreter's solution (Table 10). The petri dish was placed on the stage of the microscope. In one smooth motion, the needle was lowered until it pierced the chorion and yolk, and the pedal of the microinjector was depressed, delivering the CRISPR solution, and withdrawing slowly. For best results, the solution was injected as close to the blastodisc as possible. When the blastodisc was not visible, the solution was spread throughout the embryo by depressing the pedal while simultaneously withdrawing the needle smoothly. To reduce

Table 10. Name, type of material, company, catalog number (if applicable) of materials and equipment used in microinjection protocol for CRISPR/Cas9 in channel catfish, *Ictalurus punctatus.*

Name of Material/ Equipment	Type	Company	Catalog Number	Comments/Description
Reproboost® implant	Hormone	Center of Marine Biotechnology		Luteinizing hormone releasing hormone analog (LHRHa) for artificial spawning
TRICAINE-S	Anesthesia	Western Chemical. Inc.		For sedation of brood stock fish during hormone injection and egg stripping.
Phenol red	Reagent	Sigma-Aldrich	P0290	0.5%, sterile filtered
Stereo microscope	Equipment	Olympus	213709	For visualizing the eggs during microinjection
Microinjector	Equipment	ASI-Applied Scientific Insturmentation	Model MPPI-3	For the delivery of the injection material into the embryos
Micromanipulator	Equipment	ASI-Applied Scientific Insturmentation	Model MM33	For holding and controlling the movement of the injection needle.
Eppendorf Microloader	Tool	Eppendorf	5242956.003	For loading injection solution into microinjection needles.
Vertical needle puller	Equipment	David Kopf Instruments	Model 720	For pulling microinjection needles
Borosilicate glass capillaries	Tool	Fisher Scientific		1 mm outer diameter (OD), for making microinjection needles.
Petri dish	Tool	VWR	25384-302	For holding the embryos during the microinjection.
Crisco®	Vegetable shortening	The J.M. Smucker Company		For coating spawning pans and petri dishes.
Holtfreter`s solution	Reagent	Lab Made		59 mM NaCl, 0.67 mM KCl, 2.4 mM $NaHCO_3$, 0.76 mM $CaCl_2$, 1.67 mM $MgSO_4$ (Armstrong et al., 1989) to incubate the microinjected embryos till hatch.
Doxycycline hyclate USP (monohydrate)	Antibiotic	Letco Medical	690904	Added to Holtfreter's solution to 10 ppm to prevent bacterial infections.

mosaicism, embryos were injected between 15 min and 90 min post-fertilization, while they remained in the one cell stage. Injection controls were injected with 50nl of phenol red, while non-inject controls were not injected.

Embryos were placed in four-liter tubs of Holtfreter's solution with 10 mg/L doxycycline kept at 27°C with continuous aeration. The solution was changed, and dead embryos were removed daily. After about 5 days, or when the embryos were moving rapidly within the egg membrane and close to hatch, doxycycline treatment was discontinued. At 20 dph fry were moved to aquaria in recirculating systems until large enough to be PIT (Passive Integrated Transponders) tagged and moved to earthen ponds.

2.3. Mutation Analysis

Pelvic fin-clip samples (10-20mg) were collected in sterile 1.5mL Eppendorf tubes and kept in a -80°C freezer until DNA extraction. Genomic DNA was extracted using proteinase K digestion and ethanol precipitation using the following protocol: fin clips were digested in 600µl of cell lysis buffer (100mM NaCl, 10mM Tris, 25mM EDTA, 0.5% SDS) and 2.5µl of proteinase K in a 55°C hot water bath for 4-8 hours, with occasional vortexing. Protein was precipitated by adding 200µl of protein precipitation solution (Qiagen, 19300 Germantown Road Germantown, MD 20874), vortexed, stored on ice for 12 minutes and centrifuged for 8 minutes at 15,000rcf. The supernatant containing DNA was then precipitated with isopropanol followed by centrifugation for 5 minutes at 15,000rcf and finally washed twice with 75% ethanol by inverting gently 5-times before being dissolved in dH_2O. DNA concentration and purity was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and concentration was adjusted to 500ng/µl.

The primer set MC4R1-F, MC4R1-R (Table 9) was designed using Primer3plus to encapsulate all possible mutation sites. The Expand High Fidelity^{PLUS} PCR System (Roche) was used with 500ng of genomic DNA. A Bio-Rad T100 Thermal Cycler was used to run PCR with an initial denaturing at 95°C for 3 min; 34 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 40 sec with a ramp speed of -0.2°C/sec, extension at 72°C for 40 sec; and final extension at 72°C for 10 min.

The PCR product was confirmed on a 1% TAE Tris base, acetic acid and EDTA agarose gel. The PCR product from the treatment fish was mixed with PCR product from a wild type control of the same family at a 1:1 ratio. The combined product was then hybridized in a BioRad Thermocycler using an initial denaturing at 95°C for 3 min; 85°C for 1 min with a ramp speed of -0.2°C/sec, 75°C for 1 min with a ramp speed of -0.2°C/sec, 65°C for 1 min with a ramp speed of -0.2°C/sec, 55°C for 1 min with a ramp speed of -0.2°C/sec, 45°C for 1 min with a ramp speed of -0.2°C/sec, 35°C for 1 min with a ramp speed of -0.2°C/sec, 25°C for 1 min with a ramp speed of -0.2°C/sec.

The Surveyor® mutation detection kit (Integrated DNA Technologies) was used to detect mutations. Hybridized products were mixed with Nuclease S, Enhancer S, MgCl2 and Reaction Buffer (2) according to kit instructions and incubated at 42°C for one hour. The digested products were separated on a 1.5 percent TBE (Tris borate EDTA) agarose gel and compared with that of control samples.

To confirm and identify the mutations, positive samples were sequenced using the TA cloning method. The largest individuals from each treatment that repeatedly were confirmed as mutants using Surveyor Analysis were sequenced. First, genomic DNA from three mutants per treatment was amplified with PCR using Expand High Fidelity^{PLUS} PCR System (Roche) using the above protocol. The PCR product was verified using a 1 percent TAE agarose gel and cloned into the TOPO® TA Cloning® Kit (Invitrogen) with 20 clones per sample and sent to MCLabs for sequencing. The resulting sequences were interpreted using the MAFFT sequence alignment tool.

2.4. Generation of F1 progeny

In 2016 and 2017 spawning of MC4R broodstock was unsuccessful, even though broodstock showed outstanding secondary sexual traits. Male and female MC4R mutants were paired in aquaria and injected with 100µg/kg body weight of luteinizing hormone releasing hormone analog (LHRHa) implant in our standard procedure.

In 2018 both male and female P1 MC4R mutants were injected with 40µg/kg LHRHa and 1600 international units (IU) HCG intraperitoneally, resulting in successful spawning of all pairs that were mated. A total of 8 MC4R pairings were generated. Five pairings were made between wild-type females x P1 MC4R mutant males, 2 pairings between P1 MC4R mutant females x wild-type males and 1 pairing between a P1 MC4R mutant female x P1 MC4R mutant male. One pair of wildtype Kansas strain channel catfish from the same family were paired in identical conditions as controls but using our standard protocol of 100µg/kg LHRHa. The system received flow through water from a source pond between 26-28°C and dissolved oxygen was maintained above 5 mg/L using an air stone diffuser. Starting 24 hours after injection, the bottom of the aquaria were checked

every two hours for courtship behavior and egg masses. Egg masses were weighed and transferred to 4L bins of Holtzfreter's solution and maintained according to the protocol in 2.2.

In 2019, two more families of MC4RxMC4R and MC4RxCNTRL were generated using the same method as 2018. Two and three-year old MC4R gene edited channel catfish with good secondary sexual characteristics were chosen as brood stock. Both male and female P1 MC4R mutants were injected with 40µg/kg LHRHa and 1600 international units (IU) HCG intraperitoneally and kept in 70-liter glass aquaria. Two pairs of wild-type Kansas strain channel catfish from the same family were paired in identical conditions as controls. The system received flow through water from a source pond between 26-28°C and dissolved oxygen was maintained above 5 mg/L using an air stone diffuser. Starting 24 hours after injection, the bottom of the aquaria were checked every two hours for courtship behavior and egg masses. Egg masses were transferred to 4L bins of Holtzfreter's solution and maintained according to the protocol in 2.2.

2.5. Grow out and growth sampling

One-hundred fry per genetic type were stocked into 3-replicate 50L aquaria in recirculating systems for growth experiments. Fish in each aquarium were fed ad-libitum with Aquamax powdered and pelleted fish diets and catfish diets.

Pellet feed size was adjusted to a maximum of ¼ the size of the mouth as the fish grew. Fry were fed Purina® AquaMax® powdered starter feed until they were large enough to eat Purina® AquaMax® 100. Both feeds contained 50% protein. All fish were fed every day to satiation. Fish were weighed at 4 months, pit tagged and stocked communally in a 0.04-ha pond at

approximately 10,000 fish/ha in 3 replicate confined ponds. Fifteen fish per genetic type remained in the aquaria in the recirculating system. The fish were sampled at 10 months in both aquaria and ponds. At 15 months of age fish in ponds and in aquaria were harvested and body weight determined. During these growth trials any mortality due to disease was identified by family, genotype and sex, and the pathogen identified. Having fish in multiple environments is necessary to determine any genotype-environment interactions to ensure increased performance is observed in a more commercial-like culture unit.

2.6. Statistical analysis

To calculate differences in body weight between MC4R mutants and controls, a paired t-test was performed using R programming language (R Core Team, Vienna, Austria). To calculate differences in body weight between F1 MC4R families and CNTRL families a one-way ANOVA and Tukey's multiple comparisons test were performed using R programming language. In cases where different treatments were kept in separate aquaria at varying densities, a regression based on density was calculated, and weights were adjusted accordingly before running the statistical analysis. Differences in mutation rate were calculated with logistic regression using R programming language.

3. Results

3.1. Growth

A total of 6 MC4R mutant P1 channel catfish and 29 control channel catfish generated in 2017 were PIT tagged and transferred into a 0.04-ha earthen pond at 12-months post hatch (mean body weight for MC4R and CNTRL were 55.75g and 46.90g, respectively). Mutants and controls were kept communally with channel catfish, blue catfish, and channel catfish female x blue catfish male hybrids, to bring the density to a commercial level of 10,000 fish/ha and fed daily to satiation. At 12-months post hatch, MC4R mutants had an observed mean 19% and 8.85g larger than controls, but not significantly ($p= 0.605$, $r^2= 0.009$) (Table 11). At 18-months post hatch and market size, MC4R mutants had an observed mean 48% and 199.10g larger than controls, but not significantly ($p=0.061$, $r^2=0.103$) (Figure 11). At 27-months post hatch, the observed mean for MC4R mutants was 14% and 126.50g larger than controls, but not significantly ($p= 0.330$, $r^2= 0.050$). At 33-months post hatch, MC4R mutants had an observed mean 13% and 188.00g larger than controls, but not significantly ($p= 0.400$, $r^2= 0.038$). At 36-months post hatch, the fish were nearly too large for processor acceptability, and the observed mean for MC4R mutants was16% and 300.00g larger than controls, but not significantly ($p= 0.231$, $r^2= 0.128$).

A total of 21 MC4R female x MC4R male homozygous/bi-allelic MC4R mutant F1 channel catfish (MC4RxMC4R), 113 CNTRL female x MC4R male heterozygous MC4R mutant channel catfish in 5 families (CNTRLxMC4R-1, CNTRLxMC4R-2, CNTRLxMC4R-3, CNTRLxMC4R-4, CNTRLxMC4R-5), 36 MC4R female x CNTRL male heterozygous MC4R mutant channel catfish in 2 families (MC4RxCNTRL-1 and MC4RxCNTRL-2) and 20 control (CNTRLxCNTRL) channel catfish were generated in 2018. Mutants and controls were kept separately in 50L aquaria

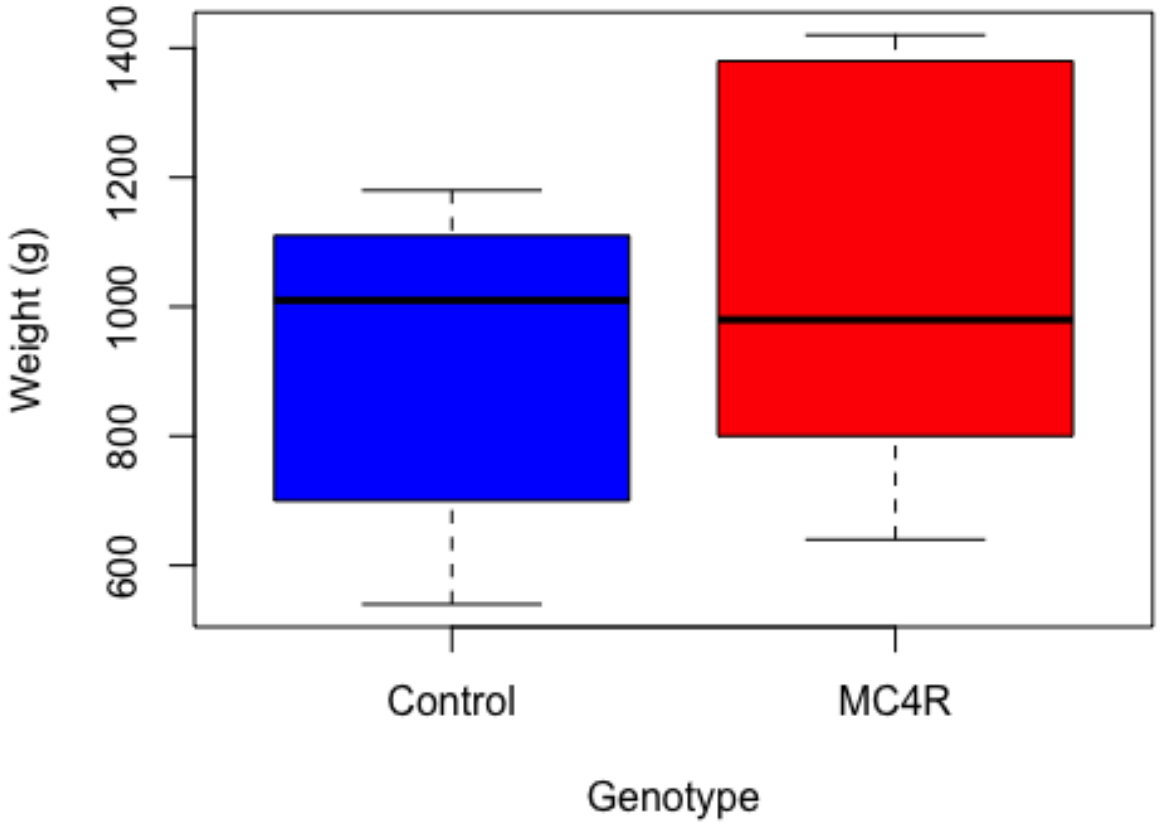


Figure 11. Box and whisker plot of body weights (grams) of control (blue) and melanocortin-4 receptor (MC4R) P1 knockout (red) channel catfish, *Ictalurus punctatus*, generated in 2017, at 27-months post hatch. Mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 0.04ha earthen pond and kept communally with unrelated channel catfish, blue catfish, *I. furcatus*, and hybrids to bring the density to a commercial level of 10,000 fish/ha and fed daily to satiation. Sampling was performed with partial seining of the pond. The plot shows median of the data (dark line), the lower and upper quartiles (25% and 75%; top and bottom of the box respectively), the area 1.5 times the interquartile range (whiskers), and outlier (circle). Paired t-test was used to determine significant differences between treatments. MC4R mutants (N=29) were 14% and 126.50g larger on average than controls (N=6) (p= 0.330).

Table 11. Mean body weights (grams), sample size (N), standard deviation (SD) and coefficient of variation (CV) of P1 melanocortin-4 receptor (MC4R) knockout mutant and control (CNTRL) channel catfish, *Ictalurus punctatus*, generated in 2017, at 12 months, 18 months, 27 months, 33 months and 36 months post hatch. Mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 0.04ha earthen pond and kept communally with unrelated channel catfish, blue catfish, *I. furcatus*, and hybrids to bring the density to a commercial level of 10,000 fish/ha and fed daily to satiation. Sampling was performed with partial seining of the pond. Paired t-test was used to determine significant p-values (<0.05).

Mean Body Weight (grams)

	12 months				18 months				27 months				33 months				36 months			
	N	Mean	SD	CV	N	Mean	SD	CV	N	Mean	SD	CV	N	Mean	SD	CV	N	Mean	SD	CV
CNTRL	27	46.90	23.45	50.01	29	417.59	217.48	52.08	16	917.50	212.78	23.19	15	1412.00	368.90	26.13	8	1820.00	305.75	16.80
MC4R	2	55.75	13.79	24.73	6	616.67	281.54	45.66	5	1044.00	346.81	33.22	6	1600.00	629.32	39.33	5	2120.00	556.06	26.23
p-value		0.522				0.151				0.475				0.517				0.314		

until 6-months post hatch when they were large enough to be PIT tagged and transferred into a communal 0.04-ha pond. Mutants and controls were kept communally with channel catfish, blue catfish, and hybrid catfish, at a maximum density of 10,000 fish/ha and fed daily to satiation. Significant differences in body weight existed among treatments at 6-months post hatch ($p<2.2e-16$). At 6-months post hatch, MC4RxMC4R mutants were 7% and 1.186g larger than CNTRLxCNTRL, but not significantly ($p=0.451$) (Table 12). CNTRLxMC4R-1 mutants were 20% and 2.75g smaller than CNTRLxCNTRL ($p=0.046$). CNTRLxMC4R-2 mutants were 12% and 1.99g larger than CNTRLxCNTRL, but not significantly ($p=0.232$). CNTRLxMC4R-3 mutants were 3% and 0.54g smaller than CNTRLxCNTRL, but not significantly ($p=0.739$). CNTRLxMC4R-4 mutants were 86% and 14.31g larger than CNTRLxCNTRL ($p=3.11e-15$). CNTRLxMC4R-5 mutants were 14% and 8.86g smaller than CNTRLxCNTRL ($p=1.26e-7$). Overall, at 6-months post hatch, CNTRLxMC4R mutants were 1% and 0.15g smaller on average than controls, but not significantly ($p= 0.934$). MC4RxCNTRL-1 mutants were 18% and 2.55g smaller than CNTRLxCNTRL, but not significantly ($p=0.111$). MC4RxCNTRL-2 mutants were 14% and 2.07g smaller than CNTRLxCNTRL, but not significantly ($p=0.222$). Overall, at 6-months post hatch, MC4RxCNTRL mutants were 16% and 2.34g smaller on average than controls, but not significantly ($p= 0.253$).

Significant differences in body weight existed among treatments at 12-months post hatch ($p<2e-16$). At 12-months post hatch, MC4RxMC4R mutants were 3% and 3.21g larger than CNTRLxCNTRL, but not significantly ($p=0.986$) (Table 12). CNTRLxMC4R-1 mutants were 38% and 8.94g smaller than CNTRLxCNTRL ($p=0.001$). CNTRLxMC4R-2 mutants were 12% and 3.86g larger than CNTRLxCNTRL, but not significantly ($p=0.277$). CNTRLxMC4R-3

Table 12. Mean body weights (grams), sample size (N), standard deviation (SD) and coefficient of variation (CV) of control, melanocortin-4 receptor (MC4R) F1 heterozygous knockout and MC4R F1 homozygous/bi-allelic knockout channel catfish, *Ictalurus punctatus*, generated in 2018 at 6-months, 12-months, 15-months, 21-months, 24-months post hatch and 28-months post hatch. Mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 0.04ha earthen pond and kept communally with unrelated channel catfish, blue catfish, *I. furcatus,* and hybrids to bring the density to a commercial level of 10,000 fish/ha and fed daily to satiation. Sampling was performed with partial seining of the pond. ANOVA was used to determine significant differences between treatments ($p<0.05$) indicated by asterisk in each column.

Mean Body Weight (grams)

	6 months				12 months				15 months				21 months				24 months				28 months			
	N	Mean	SD	CV	N	Mean	SD	CV	N	Mean	SD	CV	N	Mean	SD	CV	N	Mean	SD	CV	N	Mean	SD	CV
MC4RxMC4R	21	17.79	5.38	30.25	18	35.93	9.51	26.47	6	194.00	35.51	18.31	8	572.50	154.16	26.93	5	822.00	182.26	22.17	7	1322.86	364.31	27.54
CNTRLxMC4R-1	42	*13.85	4.69	33.85	41	*23.78	10.18	42.79	26	*148.71	52.45	35.27	24	475.83	166.50	34.99	18	583.33	263.17	45.11	21	960.00	294.75	30.70
CNTRLxMC4R-2	17	18.59	7.09	38.16	13	36.58	13.21	36.11	10	161.70	44.38	27.45	5	556.00	181.33	32.61	6	648.33	233.96	36.09	6	956.67	126.12	13.18
CNTRLxMC4R-3	18	16.06	4.21	26.25	14	26.64	5.85	21.94	9	*137.17	33.33	24.30	7	411.43	131.08	31.86	6	380.00	212.41	55.90	7	942.86	307.12	32.57
CNTRLxMC4R-4	17	*30.91	7.01	22.68	15	*55.9	14.93	26.70	5	278.10	41.14	14.79	-	-	-	-	-	-	-	-	4	1030.00	529.28	51.39
CNTRLxMC4R-5	19	*7.74	2.83	36.58	18	*17.78	5.78	32.50	13	*129.23	29.81	23.07	9	462.22	156.35	33.83	5	564.00	213.26	37.81	6	1070.00	134.31	12.55
MC4RxCNTRL-1	20	14.05	4.01	28.51	19	30.32	8.68	28.62	16	166.31	33.97	20.42	13	540.00	162.48	30.09	14	664.29	234.58	35.31	14	1148.57	324.46	28.25
MC4RxCNTRL-2	16	14.53	5.09	35.01	14	29.82	8.83	29.62	6	157.92	27.33	17.31	6	399.50	63.67	15.94	-	-	-	-		-	-	-
CNTRLxCNTRL	20	16.60	4.38	26.40	18	32.72	7.26	22.18	7	204.14	37.81	18.52	12	578.33	149.78	25.90	7	754.29	114.14	15.13	10	1044.00	304.89	29.20
MC4RxMC4R	21	17.79	5.38	30.25	18	35.93	9.51	26.47	6	194.00	35.51	18.31	8	572.50	154.16	26.93	5	822.00	182.26	22.17	7	1322.86	364.31	27.54
CNTRLxMC4R	113	16.45	8.61	52.32	101	29.52	15.94	53.98	63	155.37	57.02	36.70	45	472.00	160.25	33.95	35	556.86	249.08	44.73	44	978.18	280.92	28.72
MC4RxCNTRL	36	14.26	4.46	31.25	33	30.11	8.61	28.59	22	164.02	31.88	19.44	14	524.29	166.81	31.82	14	664.29	234.58	35.31	14	1148.57	324.46	28.25
CNTRLxCNTRL	20	16.60	4.38	26.40	18	32.72	7.26	22.18	7	204.14	37.81	18.52	12	578.33	149.78	25.90	7	754.29	114.14	15.13	10	1044.00	304.89	29.20

mutants were 23% and 6.08g smaller than CNTRLxCNTRL, but not significantly (p=0.081). CNTRLxMC4R-4 mutants were 71% and 23.18g larger than CNTRLxCNTRL (p=1.65e-10). CNTRLxMC4R-5 mutants were 84% and 14.94g smaller than CNTRLxCNTRL (p=7.85e-6). Overall, at 12-months post hatch, CNTRLxMC4R mutants were 11% and 3.20g smaller on average than controls, but not significantly (p= 0.356). MC4RxCNTRL-1 mutants were 8% and 2.41g smaller than CNTRLxCNTRL, but not significantly (p=0.452). MC4RxCNTRL-2 mutants were 10% and 2.90g smaller than CNTRLxCNTRL, but not significantly (p=0.403). Overall, at 12-months post hatch, MC4RxCNTRL mutants were 9% and 2.62g smaller on average than controls, but not significantly (p= 0.509).

Significant differences in body weight existed among treatments at 15-months post hatch (p<2.67e-8). At 15-months post hatch, MC4RxMC4R mutants were 5% and 10.14g smaller than CNTRLxCNTRL, but not significantly (p=0.999) (Table 12). CNTRLxMC4R-1 mutants were 37% and 55.43g smaller than CNTRLxCNTRL (p=0.049). CNTRLxMC4R-2 mutants were 26% and 42.44g smaller than CNTRLxCNTRL, but not significantly (p=0.475). CNTRLxMC4R-3 mutants were 49% and 66.98g smaller than CNTRLxCNTRL (p=0.041). CNTRLxMC4R-4 mutants were 36% and 73.96g larger than CNTRLxCNTRL, but not significantly (p=0.064). CNTRLxMC4R-5 mutants were 58% and 74.91g smaller than CNTRLxCNTRL (p=0.005). Overall, at 15-months post hatch, CNTRLxMC4R mutants were 32% and 48.77g smaller on average than controls , but not significantly (p= 0.077). MC4RxCNTRL-1 mutants were 23% and 37.83g smaller than CNTRLxCNTRL, but not significantly (p=0.519). MC4RxCNTRL-2 mutants were 30% and 46.23g smaller than CNTRLxCNTRL, but not significantly (p=0.525). Overall, at

15-months post hatch, MC4RxCNTRL mutants were 24% and 40.12g smaller on average than controls, but not significantly (p= 0.262).

There were no significant differences in body weight among treatments at 21-months post hatch when treatments were at market size (p=0.109) (Figure 12). Significant differences in body weight existed among treatments at 24-months post hatch (p=0.039). At 24-months post hatch,

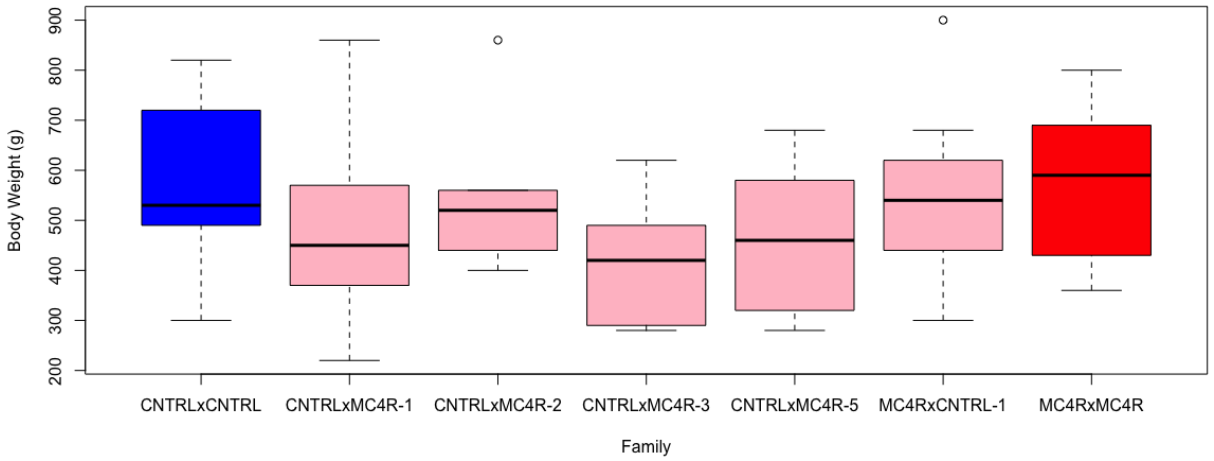


Figure 12. Box and whisker plot of body weights (grams) of control (CNTRL) (blue), melanocortin-4 receptor (MC4R) F1 heterozygous knockout (pink) and MC4R F1 homozygous/bi-allelic knockout (red) channel catfish, *Ictalurus punctatus*, generated in 2018, at 21-months post hatch. Mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 0.04ha earthen pond and kept communally with unrelated channel catfish, blue catfish, *I. furcatus*, and hybrids to bring the density to a commercial level of 10,000 fish/ha and fed daily to satiation. Sampling was performed with partial seining of the pond.The plot shows median of the data (dark line), the lower and upper quartiles (25% and 75%; top and bottom of the box respectively), the area 1.5 times the interquartile range (whiskers), and outlier (circle). ANOVA was used to determine significant differences between treatments. There were no significant differences in body weight between any treatments (p=0.109).

MC4RxMC4R mutants were 9% and 67.71 larger than CNTRLxCNTRL(p=0.999) (Table 12). CNTRLxMC4R-1 mutants were 29% and 170.95g smaller than CNTRLxCNTRL (p=0.625). CNTRLxMC4R-2 mutants were 16% and 105.95g smaller than CNTRLxCNTRL (p=0.980). CNTRLxMC4R-3 mutants were 98% and 374.29g smaller than CNTRLxCNTRL (p=0.064). CNTRLxMC4R-4 mutants were not measured during this sampling period, due to partial seining of the pond. CNTRLxMC4R-5 mutants were 34% and 190.29g smaller than CNTRLxCNTRL (p=0.783). Overall, at 24-months post hatch, CNTRLxMC4R mutants were 36% and 197.43g smaller on average than controls (p= 0.177). MC4RxCNTRL-1 mutants were 14% and 90.00g smaller than CNTRLxCNTRL (p=0.977). MC4RxCNTRL-2 mutants were not measured during this sampling period. Overall, at 24-months post hatch, MC4RxCNTRL mutants were 14% and 90.00g smaller on average than controls (p= 0.833).

No significant differences in body weight existed among families at 28-months post hatch (p=0.196). However, homozygous/bi-allelic mutants were 30% larger than pooled F1 heterozygotes (p=0.022).

A total of 112 MC4R female x MC4R male homozygous/bi-allelic MC4R mutant F1 channel catfish (MC4RxMC4R), 109 MC4R female x CNTRL male heterozygous MC4R mutant channel catfish (MC4RxCNTRL), and 108 control channel catfish in two families (CNTRLxCNTRL-1 and CNTRLxCNTRL-2) were generated in 2019. Mutants and controls were kept separately in 50L aquaria until 6-months post hatch when they were large enough to be PIT tagged and transferred into a communal 0.04-ha pond. Mutants and controls were kept communally with channel catfish, blue catfish, and hybrid catfish, at a maximum density of 10,000 fish/ha and

fed daily to satiation. Significant differences in body weight existed among treatments at 12-months post hatch (p=1.57e-7) (Table 13). At 12-months post hatch, MC4RxMC4R mutants were 30% and 8.77g larger than the mean of both CNTRLxCNTRL families (p=7.1e-5) (Table 13). MC4RxCNTRL mutants were 9% and 2.34g smaller than CNTRLxCNTRL (p=0.493).

Significant differences in body weight existed among treatments at 18-months post hatch (p=1.68e-4). At 18-months post hatch, MC4RxMC4R mutants were 40% and 15.75g larger than the mean of both CNTRLxCNTRL families (p=0.005) (Figure 13). MC4RxCNTRL mutants were 10% and 3.73g smaller than CNTRLxCNTRL (p=0.721)

3.2. Mutation analysis

A total of 18 fish survived microinjection of CRISPR/Cas9 and sgRNA targeting exon 1 of the MC4R gene in 2017. The mutation rate of the survivors was 33.3% (6/18) (Table 14). There was a significant difference in mutation rate between families generated in 2018 ($p<0.05$). Of the 6 P1 mutants 33.3% were homozygous/bi-allelic and 66.7% were heterozygous. In 2018, a total of 398 F1 MC4R offspring within 8 families were generated by pairing P1 mutants. One family was generated by pairing a MC4R mutant female with a MC4R mutant male (MC4RxMC4R), 5 families were generated by pairing a wild-type female with a MC4R mutant male (CNTRLxMC4R-1,2,3,4,5), and 2 families were generated by pairing a MC4R mutant female with a wild-type male (MC4RxCNTRL-1,2). The average mutation rate for all F1 MC4R channel catfish was 42% (170/398). Of the 21 MC4RxMC4R F1 mutants, 76% (16/21) were homozygotes/bi-allelic.

Table 13. Mean body weights (grams), sample size (N), standard deviation (SD) and coefficient of variation (CV) of melanocortin-4 receptor (MC4R) F1 heterozygous knockout, MC4R F1 homozygous/bi-allelic knockout and control (CNTRL), channel catfish, *Ictalurus punctatus*, generated in 2019 at 12-months and 18-months post hatch. Mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 0.04ha earthen pond and kept communally with unrelated channel catfish, blue catfish, *I. furcatus*, and hybrids to bring the density to a commercial level of 10,000 fish/ha and fed daily to satiation. Sampling was performed with partial seining of the pond. ANOVA was used to determine significant differences between CNTRLxCNTRL-2 ($p<0.05$) indicated by asterisk in each column.

Mean Body Weight (grams)

	12 months				18 months			
	N	Mean	SD	CV	N	Mean	SD	CV
MC4RxMC4R	112	*37.58	10.07	26.79	41	*55.44	21.18	38.21
MC4RxCNTRL	109	26.47	8.94	33.76	44	35.95	17.10	47.56
CNTRLxCNTRL-1	79	30.49	25.98	85.19	26	43.88	29.30	66.76
CNTRLxCNTRL-2	30	24.37	9.01	36.96	12	30.58	18.24	59.63
MC4RxMC4R	112	*37.58	10.07	26.79	41	*55.44	21.18	38.21
MC4RxCNTRL	108	26.39	8.94	33.88	44	35.95	17.10	47.56
CNTRLxCNTRL	109	28.81	22.73	78.91	38	39.68	26.80	67.52

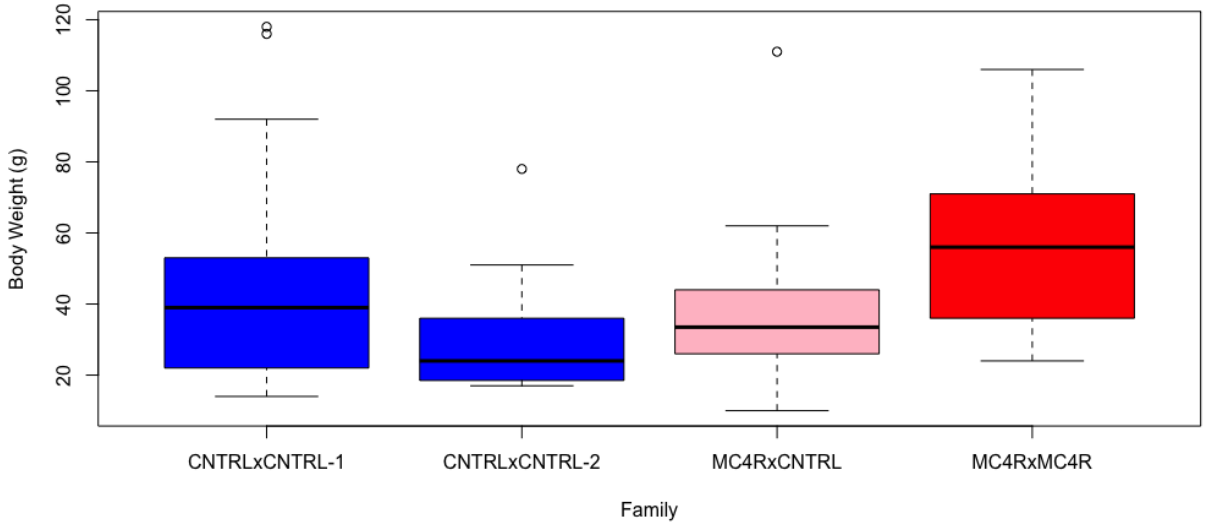


Figure 13. Box and whisker plot of body weights (grams) of control (blue), melanocortin-4 receptor (MC4R) F1 heterozygous knockout (pink) and MC4R F1 homozygous/bi-allelic knockout (red) channel catfish, *Ictalurus punctatus*, generated in 2019 at 18-months post hatch. Mutants and controls were kept separately in 50L aquaria until 12-months post hatch, when they were pit-tagged and transferred to a 0.04ha earthen pond and kept communally with unrelated channel catfish, blue catfish, *I. furcatus*, and hybrids to bring the density to a commercial level of 10,000 fish/ha and fed daily to satiation. Sampling was performed with partial seining of the pond. The plot shows median of the data (dark line), the lower and upper quartiles (25% and 75%; top and bottom of the box respectively), the area 1.5 times the interquartile range (whiskers), and outlier (circle). ANOVA was used to determine significant differences between treatments. At 18-months post hatch there was a significant difference in body weight between families (p=1.68e-4)

Table 14. Mutation rate and zygosity of all melanocortin-4 receptor (MC4R) gene edited channel catfish, *Ictalurus punctatus*, generated in 2017, 2018 and 2019. Each row indicates a different family. P1 indicates fish gene edited through microinjection using CRISPR/Cas9 and various gRNAs targeting exon one of the MC4R gene. Approximately 50nl of solution, composed of 300-350ng/µl Cas9 protein and 0-200ng/µl sgRNA, was injected into each embryo close to the blastodisc 15 min after fertilization using a glass capillary needle. F1 indicates the offspring of either control channel catfish female x control channel catfish male, control channel catfish female x homozygous/bi-allelic MC4R mutant channel catfish male, homozygous/bi-allelic MC4R mutant channel catfish female x control channel catfish or MC4R mutant channel catfish female x homozygous/bi-allelic MC4R mutant channel catfish male. Logistic regression was used to determine significant p-values (<0.05). There were significant differences in mutation rate between families in 2018 and 2019.

Year Spawned	Family	Mutation Rate KO	Homozygosity/Bi-allelism
2017	P1 MC4R Channel	33% (6/18)	33% (2/6)
2018	F1 MC4RxMC4R	50% (21/42)	76% (16/21)
2018	F1 CNTRLxMC4R-1	67% (42/63)	0% (0/42)
2018	F1 CNTRLxMC4R-2	44% (17/39)	0% (0/17)
2018	F1 CNTRLxMC4R-3	50% (18/36)	0% (0/18)
2018	F1 CNTRLxMC4R-4	50% (17/34)	0% (0/17)
2018	F1 CNTRLxMC4R-5	83% (19/23)	0% (0/19)
2018	F1 MC4RxCNTRL-1	15% (20/133)	0% (0/20)
2018	F1 MC4RxCNTRL-2	57% (16/28)	0% (0/16)
2018	CNTRLxCNTRL	0% (0/20)	0% (0/0)
2019	F1 MC4RxMC4R	86% (112/130)	72% (81/112)
2019	F1 MC4RxCNTRL	61% (109/180)	0% (0/109)

In 2019, a total of 130 F1 MC4R offspring were generated by pairing a MC4R mutant female with a MC4R mutant male (MC4RxMC4R). The mutation rate was 86% (112/130) and 72% of mutants were homozygotes/bi-allelic (81/112) (Table 14). The same year, a total of 180 F1 MC4R offspring were generated by pairing a MC4R mutant female with a wild-type male (MC4RxCNTRL). The mutation rate was 61% (109/180). The average mutation rate for all F1 MC4R channel catfish was 71% (221/310). There was a significant difference in mutation rate between families generated in 2019 ($p<0.05$).

Gel electrophoresis confirmed mutations in both P1 and F1 generations of MC4R mutants (Figure 14). Multiple bands in the image corresponded to expected cut sites. In all cases, 1 band indicated a wild-type sequence, while multiple bands were associated with mutations. Each positive result was confirmed with a second gel.

Sequence results confirmed mutations indicated by gel electrophoresis. Two F1 mutants were tested with each possessing mutations in exon 1 in the MC4R gene (Figure 15). Both samples had large deletions occurring outside of the target sites. There were no insertions generated. The MC4RxMC4R sample was a homozygous/bi-allelic mutant and contained a deletion (-641bp) spanning nearly the entire amplicon. The CNTRLxMC4R sample was a heterozygous mutant with four large deletions (-427bp, -211bp, -67bp, -127bp) and 26 substitutions.

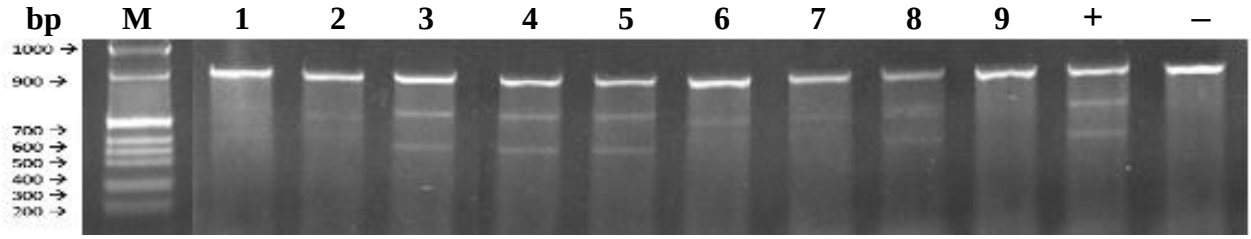


Figure 14. Identification of edited melanocortin-4 receptor (MC4R) gene sequences in channel catfish, *Ictalurus punctatus*, using the surveyor mutation detection assay. All samples were hybridized with an equal volume of non-injected control (Sample –) to detect both homozygotes/bi-allelic and heterozygotes. Wild-type sequences are indicated with a single 932 bp band, while mutations are signified by three bands. Samples 1 and 2 are MC4R x MC4R F1 progeny. Sample 3 is CNTRL x MC4R-1 F1 progeny. Samples 4 and 5 are CNTRL x MC4R-2 F1 progeny. Samples 6 and 7 are CNTRL x MC4R-3 F1 progeny. Samples 8 and 9 are CNTRL x MC4R-4 F1 progeny. Sample + is a previously identified MC4R x MC4R F1 progeny mutant. Sample – came from wild-type control. M indicates 1kb marker.

Wild-type

ggagatggaggacacggaagagactcgcagattagaataaacgcag**ATG**AAGACGGAAAGCGGAGGACTGTGGTGAGGAGGTCTTGCGGATATGAACGTGTC
GGAGCACCACGGGATGCAGCATGCACACCGGAACCACAGCCTGGGCGTGCAGATTGGAAACAAAGCCGGCTCGGGGGAAAGGAACT
CGGAGTCGGGCTGCTACGAGCAGCTGTTGATCTCCACCGAGGTCTTCATCACGCTAGGGTTGGTCAGCCTTCTGGAGAACATCCTGGT
AATCGCGGCCATCGTCAAGAACAAGAACTTCCACTCGCCCATGTACTTCTTCATCTGCAGCCTGGCGGTGGCCGACCTGCTGGTGAGC
GTATCGAACGCGACAGAAACGGCTGTGATGGCGCTGATCACCAGCGGCAACCTGACCATCTCTGGAGACGTCGTGAAAAGCATGGA
CAATGTGTTCGACTCCATGATCTGCAGCTCACTCCTGGCCTCCATTTGGAGTCTCCTGGCCATCGCCGTGGACCGCTACGTCACCATCT
TCTACGCCCTGCGCTACCACAACATCATGACCCAACGCCGCGCGGCGCTCATCATCGTATGCATATGGAGCTTCTGCACGGCGTCCGG
TGTGCTCTTCATCATCTACTCGGAGAGCGCTACAGTCCTCATCTGCCTTATCAGCATGTTCTTCACCATGCTGGCCCTCATGGCCTCGC
TTTACGTGCACATGTTCCTCTTGGCGCGGCTTCACATGAAACGCATCGCCGCCTTACCGGGGAACGGCCCCGTGTGGCAGGCGGCCAA
CATGAAGGGCGCCGTGACGCTCACCATCCTGCTCGGAGTGTTTGTCGTGTGCTGGGCGCCGTTTTTTCTCCACCTCATTCTCATGAtctctt
gtccgaggaacccgtattgcgtctgcttcatgtctc

MC4R x MC4R

Wild Sequence

5’ TGGAAACAAAGCCGGCTCGGGGGAAAGGAACTCGGAGTCGGGCTG//CAGGCGGCCAACATGA 3’
AA: GNKAGSGERNSESGCYEQLLISTEVFITLGLVSLLENILVIAAIVKNKNFHSPMYFFICSLAVADLLVSVSNATETAVMALITSGNLTISG
DVVKSMDNVFDSMICSSLLASIWSLLAIAVDRYVTIFYALRYHNIMTQRRAALIIVCIWSFCTASGVLFIIYSESATVLICLISMFFTMLALMA
SLYVHMFLLARLHMKRIAALPGNGPVWQAANM

Mutated Sequence

5’ TGGAAACAAAGCC----------/----------CAGGCGGCCAACATGA 3’ [-641]
AA: GNKAQAANM

CNTRL x MC4R-1

Wild Sequence

5’ aag//GGACAATGTGTTCGACTCC// TCAGCA// //TTCACATGAAACGCA////GAtctcttgtccgaggaacccgtat 3’
AA: MDNVFDSMICSSLLASIWSLLAIAVDRYVTIFYALRYHNIMTQRRAALIIVCIWSFCTASGVLFIIYSESATVLICLISM
FFTMLALMASLYVHMFLLARLHMKRIAALPGNGPVWQAANMKGAVTLTILLGVFVVCWAPFFLHLILMISCPRNPYCVCFMS

Mutated Sequence

5’aag--/--GAGTAGTATGTCCGAATTC--/--TCAGTA--/--TTCATATAAAGAGTA--/--GACCAGTAATACGTGGATGACCtat 3’ [-427, -211, -67, -127]
AA: SSMSEFSVFI–RVDQ–YVDDLLRLLHV 3’

Figure 15. CRISPR/Cas9 induced mutations in exon 1 of melanocortin-4 receptor (MC4R) gene coding sequence of channel catfish, *Ictalurus punctatus*, in two F1 mutants. The exons and introns are indicated by upper and lower case and the underlined bold uppercase is the start codon. The primers used in PCR are indicated in red. The guide RNA target sites are indicated in green followed by PAM (Protospacer adjacent motif, NGG) in blue. Deletion mutations are represented by a dashed line with each dash corresponding to a nucleotide that has been deleted. Double slash indicates wild-type continuation of the sequence for simplicity. Single slash indicates that there is a large deletion. Each sequence starting with 5’ and ending with 3’ came from a single reaction representing a single allele. Brackets indicate deletion/insertion/substitution value. Wild-type sequence acquired from Genbank (Accession No. LBML01001141.1). Corresponding predicted amino acid sequence indicated by “AA”. Red letters indicate substitutions. Predicted amino acid sequence acquired from Expasy and confirmed with NCBI ORFfinder.

4. Discussion

In the current study, the effects of microinjection of gRNAs targeting exon 1 of the channel catfish melanocortin-4 receptor gene in conjunction with Cas9 protein on mutation rate and growth were investigated. Efficient mutagenesis was achieved as demonstrated by PCR, Surveyor assay and DNA sequencing. A total of 18 fish survived microninjection of CRISPR/Cas9 and sgRNA targeting exon one of the MC4R gene in 2017 with a mutation rate of 33% (6/18). Of the 6 P1 mutants 33% were homozygous/bi-allelic. This is substantially lower than the mutation rate of 87% achieved in both zebrafish and rats (Xie et al., 2016; Li et al., 2013). Growth was generally, but not always, higher in P1 MC4R mutants when compared to controls at all life stages and in pond and tank environments. A hormone mix of LHRHa and HCG was necessary as a therapy and inducement to allow P1 MC4R mutant channel catfish to spawn, resulting in the F1 generation. Homozygous F1 MC4R mutants grew 26.7% faster than controls in earthen ponds to a kg, but heterozygous F1 mutants were not different than controls for body weight.

Successful spawning of MC4R mutants to produce F1 progeny was achieved in both 2018 and 2019, and offspring inherited the mutation at a high rate. In 2018, a total of 398 F1 MC4R offspring within 8 families were generated by pairing P1 mutants. The average mutation rate for all F1 MC4R channel catfish was 42% (170/398). Of the 21 MC4RxMC4R F1 mutants, 76% (16/21) were homozygotes/bi-allelic. In 2019, a total of 310 F1 MC4R offspring within 2 families were generated by pairing P1 mutants. The average mutation rate for all F1 MC4R channel catfish was 71% (221/310). The inheritance rates were much higher than those achieved by Hruscha et al. (2013) and Vashney et al. (2015), where 11% and 28% of zebrafish progeny inherited the MC4R

mutation, indicating much less mosaicism being generated with our protocols for catfish. The variability was likely due to a combination of environmental and genetic effects.

Growth was generally higher in MC4R mutants when compared to controls at all life stages and in both pond and tank environments. Beyond the stocker stage, MC4R mutants grew faster than controls. There was a positive relationship between zygosity and growth, with F1 homozygous/bi-allelic mutants growing faster than both MC4RxCNTRL and CNTRLxMC4R F1 heterozygotes. This increase in growth in MC4R mutants supports previous studies on fish, chickens, pigs and mice (Yang et al., 2018; Yang et al., 2020; Wan et al., 2012; Li et al., 2006; Kim et al., 2000; Lu et al., 1994). Homozygous F1 MC4R mutants grew 26.7% faster than controls to market size. Similarly, Holland's carp containing a SNP in the MC4R gene grew 25% faster at 1-year of age than wild-type carp (Yang, 2018). MC4R knockout zebrafish were 15.7% larger at 3-months of age than wild-type zebrafish (Fei et al., 2017; calculated using ImageJ). Chicken containing a SNP in the MC4R gene were between 1.1-6.7 %larger than wild type chickens (Li and Li, 2006; Kubota et al., 2019). Al-Thuwaini et al. (2020) found that Awassi sheep breed containing a SNP in the MC4R gene grew 17.1% faster than the Arabi breed containing the wild-type MC4R gene. Pigs containing a SNP in the MC4R gene grew 0-6.0% faster than wild-type individuals (Meidtner et al., 2006; Panda et al., 2019). While few studies have investigated mutations of MC4R in fish, current data indicates that mutations in fish result in larger phenotypic gains than terrestrial animals. The larger gains in teleost fish are likely explained by the larger proportion of muscle to body weight in fish compared to terrestrial animals (Tlusty et al., 2018) or the indeterminate growth of fish. This faster growth to harvest makes MC4R a valuable gene commercially.

The growth of individual MC4R mutants and different MC4R families varied. This is likely due to differences in knockout patterns and subsequent expression levels of MC4R, differences in genetic background coupled with epistatic interaction with other loci. The variability can be advantageous as combining gene editing with selection for the largest individuals might result in maximum genetic enhancement.

There appeared to be an effect of zygosity on body weight. The wild-type allele appears to be dominant as body weight in heterozygous MC4R mutants was not different than that of controls, while homozygous mutants were generally larger than controls. This is the opposite of previous studies that found mutations in MC4R in humans are associated with a dominant form of obesity (Vaisse et al., 1998; Yeo et al., 1998; Hinney et al., 1999). Similar to our results with channel catfish, chickens with bi-allelic SNPs in the G54C locus of the MC4R gene were larger than heterozygous mutants and wild-type chickens, while heterozygotes were smaller than wild-type chickens (Li and Li, 2006). Ortega-Azorin (2012) found a thir genetic mechanism in play for MC4R mutants, as there was an additive effect of human MC4R polymorphisms on appetite, while Kim et al. (2004; 2006) showed that additive action of MC4R may influence growth and fat deposition in pigs. In channel catfish, it appears that only one functional copy of the MC4R wild type allele is necessary for normal growth. Further research should evaluate whether zygosity impacts other important traits such as fertility, survival and fat deposition in channel catfish.

The improved growth indicates that the use of gene edited MC4R channel catfish could be beneficial for commercial farms. Catfish farming and production in the United States peaked in

2003, dramatically declined from 2007-2012 and has been gradually increasing since that time (Hanson and Sites, 2015; Torrans and Ott, 2018; FAO, 2020). Gene editing presents a valuable tool to increase profitability, sustainability and industry growth. There are, however, a number of ethical, logistical and regulatory hurdles for the MC4R mutant channel catfish to become applied commercially in the United States, as FDA currently regulates gene edited animals. The improvement of gene editing technologies, greater understanding of its effects and the commercial success of genetically improved organisms, including Aquabounty's AquaAdvantage salmon make this technology a viable option in the near future. By combining MC4R gene editing with other genetic techniques, such as selection, crossbreeding and hybridization, it is likely possible to achieve even greater growth results, shorten the grow-out period as well as select for multiple traits. With an increasing human population and declining natural resources, all solutions should be evaluated to determine the most efficient and sustainable methods of food production.

References

Al-Thuwaini, T. M., Al-Shuhaib, M. B. S., Lepretre, F., & Dawud, H. H. (2020). Two co-inherited novel SNPs in the MC4R gene related to live body weight and hormonal assays in Awassi and Arabi sheep breeds of Iraq. *Veterinary Medicine and Science*.

Carroll, L., Voisey, J., & Van Daal, A. (2005). Gene polymorphisms and their effects in the melanocortin system. *Peptides*, *26*(10), 1871-1885.

Cerda-Reverter J. M., Ringholm A., Schioth H. B., Peter R. E. (2003). Molecular cloning, pharmacological characterization, and brain mapping of the melanocortin 4 receptor in the goldfish: involvement in the control of food intake. *Endocrinology*. 144:2336–49.

Chen, X., Huang, L., Tan, H. Y., Li, H., Wan, Y., Cowley, M., ... & Chen, C. (2017). Deficient melanocortin-4 receptor causes abnormal reproductive neuroendocrine profile in female mice. *Reproduction*, *153*(3), 267-276.

Cuevas-Uribe, R., & Tiersch, T. R. (2011). Estimation of fish sperm concentration by use of spectrophotometry. Cryopreservation in Aquatic Species, 2nd edition. World Aquaculture Society, Baton Rouge, LA, 162-200.

Davoli, R., Braglia, S., Valastro, V., Annarratone, C., Comella, M., Zambonelli, P., ... & Russo, V. (2012). Analysis of MC4R polymorphism in Italian Large White and Italian Duroc pigs: association with carcass traits. *Meat Science*, *90*(4), 887-892.

Du, X. H., Chen, C., Yuan, Z. R., Zhang, L. M., Chen, X. J., Wang, Y. H., ... & Xu, S. Z. (2013). Genetic Polymorphisms of MC4R and IGF2 Gene Association with Feed Conversion Efficiency Traits in Beef Cattle. *Pakistan Veterinary Journal*, *33*(4).

Ergün, S., Soyutürk, M., Güroy, B., Güroy, D., & Merrifield, D. (2009). Influence of Ulva meal on growth, feed utilization, and body composition of juvenile Nile tilapia (*Oreochromis niloticus*) at two levels of dietary lipid. *Aquaculture International*, *17*(4), 355-361.

Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J., & Cone, R. D. (1997). Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature*, *385*(6612), 165-168.

Fei, F., Sun, S. Y., Yao, Y. X., & Wang, X. (2017). Generation and phenotype analysis of zebrafish mutations of obesity-related genes lepr and MC4R. *Sheng li xue bao*, *69*(1), 61-69.

Goddard, S. (2012). *Feed management in intensive aquaculture*. Springer Science & Business Media. Chapman & Hall, Dept. BC, 115 Fifth Avenue, New York, NY 10003.

Godino-Gimeno, A., Sánchez, E., Guillot, R., Rocha, A., Angotzi, A. R., Leal, E., ... & Cerdá-Reverter, J. M. (2020). Growth Performance After Agouti-Signaling Protein 1 (Asip1) Overexpression in Transgenic Zebrafish. *Zebrafish*, *17*(6), 373-381.

Hinney, A., Schmidt, A., Nottebom, K., Heibult, O., Becker, I., Ziegler, A., ... & Hebebrand, J. (1999). Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans. *The Journal of Clinical Endocrinology & Metabolism*, *84*(4), 1483-1486.

Hruscha, A., Krawitz, P., Rechenberg, A., Heinrich, V., Hecht, J., Haass, C., & Schmid, B. (2013). Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. *Development*, *140*(24), 4982-4987.

Jiang, D. N., Li, J. T., Tao, Y. X., Chen, H. P., Deng, S. P., Zhu, C. H., & Li, G. L. (2017). Effects of melanocortin-4 receptor agonists and antagonists on expression of genes related to reproduction in spotted scat, *Scatophagus argus*. *Journal of Comparative Physiology B*, *187*(4), 603-612.

Kawahara, A., Yabe, T., Ansai, S., Takada, S., & Kinoshita, M. (2015). Genome Editing in Zebrafish and Medaka. *Targeted Genome Editing Using Site-Specific Nucleases* (pp. 119-131). Springer, Tokyo.

Kim, K. S., Larsen, N., Short, T., Plastow, G., & Rothschild, M. F. (2000). A missense variant of the porcine melanocortin-4 receptor (MC4R) gene is associated with fatness, growth, and feed intake traits. *Mammalian Genome*, *11*(2), 131-135.

Kim, K. S., Thomsen, H., Bastiaansen, J., Nguyen, N. T., Dekkers, J. C., Plastow, G. S., & Rothschild, M. F. (2004). Investigation of obesity candidate genes on porcine fat deposition quantitative trait loci regions. *Obesity Research*, *12*(12), 1981-1994.

Kim, K. S., Lee, J. J., Shin, H. Y., Choi, B. H., Lee, C. K., Kim, J. J., ... & Kim, T. H. (2006). Association of melanocortin 4 receptor (MC4R) and high mobility group AT-hook 1 (HMGA1) polymorphisms with pig growth and fat deposition traits. *Animal Genetics*, *37*(4), 419-421.

Koots, K. R. (1994). Analyses of published genetic parameter estimates for beef production traits. 2. Phenotypic and genetic correlations. *Animal Breeding Abstracts* (Vol. 62, pp. 825-853).

Kubota, S., Vandee, A., Keawnakient, P., Molee, W., Yongsawatdikul, J., & Molee, A. (2019). Effects of the MC4R, CAPN1, and ADSL genes on body weight and purine content in slow-growing chickens. *Poultry Science*, *98*(10), 4327-4337.

Lampert, K. P., Schmidt, C., Fischer, P., Volff, J. N., Hoffmann, C., Muck, J., ... & Schartl, M. (2010). Determination of onset of sexual maturation and mating behavior by melanocortin receptor 4 polymorphisms. *Current Biology*, *20*(19), 1729-1734.

Lee, Y., Park, S., Kim, H., Lee, S. K., Kim, J. W., Lee, H. K., ... & Lee, S. J. (2013). A C1069G SNP of the MC4R gene and its association with economic traits in Korean native cattle (brown, brindle, and black). *Electronic Journal of Biotechnology*, *16*(5), 14-14.

Li, C. Y., & Li, H. (2006). Association of MC4R gene polymorphisms with growth and body composition traits in chicken. *Asian-Australasian Journal of Animal Sciences*, *19*(6), 763-768.

Li, D., Qiu, Z., Shao, Y., Chen, Y., Guan, Y., Liu, M., ... & Liu, M. (2013). Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. *Nature Biotechnology*, *31*(8), 681-683.

Liu, R., Kinoshita, M., Adolfi, M. C., & Schartl, M. (2019). Analysis of the role of the MC4R system in development, growth, and puberty of medaka. *Frontiers in Endocrinology*, *10*, 213.

Lu, D., Willard, D., Patel, I. R., Kadwell, S., Overton, L., Kost, T., ... & Cone, R. D. (1994). Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature*, *371*(6500), 799-802.

Mrode, R. A., Smith, C., & Thompson, R. (1990). Selection for rate and efficiency of lean gain in Hereford cattle. 2. Evaluation of correlated responses. *Animal Science*, *51*(1), 35-46.

Ortega-Azorín, C., Sorlí, J. V., Asensio, E. M., Coltell, O., Martínez-González, M. Á., Salas-Salvadó, J., ... & Corella, D. (2012). Associations of the FTO rs9939609 and the MC4R rs17782313 polymorphisms with type 2 diabetes are modulated by diet, being higher when adherence to the Mediterranean diet pattern is low. *Cardiovascular Diabetology*, *11*(1), 1-12.

Panda, S., Gaur, G. K., Sahoo, N. R., & Saini, B. L. (2019). Association of MC4R, RYR1 and PRKAG3 single nucleotide polymorphisms with body weight in crossbred piglets. *Indian Journal of Animal Sciences*, *89*(5), 539-542.

Phelps, R. P., Hastey, R., Pendetar, A., Linley, L., Papanikos, N., & Dunham, R. A. (2007). Effects of temperature on the induced spawning of channel catfish and the production of channel× blue catfish hybrid fry. *Aquaculture*, *273*(1), 80-86.

Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018.

Robinson, E. H., & Li, M. H. (2015). *Feed conversion ratio for pond-raised catfish*. Mississippi Agricultural & Forestry Experiment Station.

Schjolden, J., Schioth, HB., Larhammar, D., Winberg, S., Larson, ET. (2009). Melanocortin peptides affect the motivation to feed in rainbow trout (*Oncorhynchus mykiss*). *Gen Comp Endocrinol*. 160:134–8.

Sharma, P. M. (2005). *Polymorphisms in genes associated with feed efficiency and production traits in broilers*. University of Arkansas.

Smith, C. C., Harris, R. M., Lampert, K. P., Schartl, M., Hofmann, H. A., & Ryan, M. J. (2015). Copy number variation in the melanocortin 4 receptor gene and alternative reproductive tactics the swordtail *Xiphophorus multilineatus*. *Environmental Biology of Fishes*, *98*(1), 23-33.

Song, Y., & Cone, R. D. (2007). Creation of a genetic model of obesity in a teleost. *The FASEB Journal*, *21*(9), 2042-2049.

Tlusty, M., Tyedmers, P., Ziegler, F., Jonell, M., Henriksson, P., Newton, R., ... & Cao, L. (2018). Commentary: comparing efficiency in aquatic and terrestrial animal production systems. *Environmental Research Letters*, *13*(12).

Vaisse, C., Clement, K., Guy-Grand, B. *et al.* (1998). A frameshift mutation in human MC4R is associated with a dominant form of obesity. *Nature Genetics,* 20, 113–114.

Varshney, G. K., Pei, W., LaFave, M. C., Idol, J., Xu, L., Gallardo, V., ... & Burgess, S. M. (2015). High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. *Genome Research*, *25*(7), 1030-1042.

Wan, Y., Zhang, Y., Ji, P., Li, Y., Xu, P., & Sun, X. (2012). Molecular characterization of CART, AgRP, and MC4R genes and their expression with fasting and re-feeding in common carp (*Cyprinus carpio*). *Molecular Biology Reports*, *39*(3), 2215-2223.

Xie, S. L., Bian, W. P., Wang, C., Junaid, M., Zou, J. X., & Pei, D. S. (2016). A novel technique based on in vitro oocyte injection to improve CRISPR/Cas9 gene editing in zebrafish. *Scientific Reports*, *6*, 34555.

Yang, Y., Lan, Z., Shu, H., Zhou, H., Jiang, X., Hou, L., & Gu, P. (2018). Association between expression levels and growth trait-related SNPs located in promoters of the MC4R and MSTN genes in *Spinibarbus hollandi. Genes & Genomics*, *40*(11), 1119-1125.

Yang, Z., Liang, X. F., Li, G. L., & Tao, Y. X. (2020). Biased signaling in fish melanocortin-4 receptors (MC4Rs): Divergent pharmacology of four ligands on spotted scat (*Scatophagus argus*) and grass carp (*Ctenopharyngodon idella*) MC4Rs. *Molecular and Cellular Endocrinology*, 110929.

Yeo, G. S., Farooqi, I. S., Aminian, S., Halsall, D. J., Stanhope, R. G., & O'Rahilly, S. (1998). A frameshift mutation in MC4R associated with dominantly inherited human obesity. *Nature Genetics*, *20*(2), 111-112.

Zohar, Y., & Mylonas, C. C. (2001). Endocrine manipulations of spawning in cultured fish: from hormones to genes. In *Reproductive Biotechnology in Finfish Aquaculture* (pp. 99-136). Lee, C. S., & Donaldson, E. Elsevier. Amsterdam, The Netherlands

Chapter 4: Effect of Simultaneous Knockout of MC4R Gene and Knock-In of Elongase Gene on Channel Catfish, *Ictalurus punctatus*

Abstract

Channel catfish, *Ictalurus punctatus*, is the primary aquaculture species in the United States. However, catfish farming has dramatically declined from its peak in 2003, largely due to the import of catfish from Asia. In order to compete with foreign supply, an improved catfish was developed possessing increased growth and higher nutritional qualities. Channel catfish have limited ability to synthesize n-3 fatty acids, due to a lack of elongases and desaturases. The ccβA-msEvol2 transgene containing masu salmon, *Oncorhynchus masou*, elongase gene driven by the common carp, *Cyprinus carpio*, beta-actin promoter was inserted into the channel catfish melanocortin-4 receptor (MC4R) gene using the 2-hit 2-oligo with plasmid (2H2OP) method. Two sets of sgRNAs targeting exon 1 of the channel catfish MC4R gene in conjunction with Cas9 protein, a pair of single-stranded oligo donor (ssODNs), the ccβA-msEvol2 transgene and a sgRNA targeting the transgene were microinjected in embryos, and mutation rate, growth and omega-3 fatty acid levels were studied. Efficient mutagenesis was achieved as demonstrated by PCR, Surveyor assay and DNA sequencing. SgRNA one (MC4R-A) targeting exon one of the MC4R gene resulted in a knockout mutation rate of 92% with 69% homozygosity/bi-allelism, a knock-in rate of 54% and a simultaneous knockout/knock-in rate of 49%. SgRNA 2 (MC4R-D) targeting exon one of the MC4R gene resulted in a knockout mutation rate of 25% with 38% homozygosity/bi-allelism, a knock-in rate of 6% and no simultaneous knockout/knock-in. Evol2/MC4R fish were 41.81% larger than controls at 6-months post hatch ($p=0.005$). There was no significant increase in overall omega three content, however observed mean eicosapentaenoic

acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) content were 92.16% (p=0.203) and 32.82% (p=0.380) higher, respectively, in Evol2/MC4R fish than controls. With a high mutation rate, improved growth and higher omega-3 fatty acid content the use of Evol2/MC4R channel catfish appear to be beneficial for application on commercial farms.

Keywords: Channel catfish, *Ictalurus punctatus*, elongase, omega-3 fatty acid, melanocortin-4 receptor, MC4R, growth, gene editing, transgenesis, CRISPR/Cas9

1. Introduction

Catfish production constituted approximately 68% of the total domestic aquaculture production in 2015 (NOAA, 2016). However, catfish production has had more than a 50% reduction from 2003 (Hanson and Sites, 2015). The factors that caused the decline of catfish industry included intense competition from imported products from Asia, increased feed and fuel costs, and fish disease control problems (Wagner et al., 2002; FAO, 2020).

Growth and feed conversion efficiency are basic and important traits affecting production costs and profitability. In catfish farming, feed is 60% of the variable cost (Robinson and Li, 2015). Feed conversion is also important to optimize for environmental reasons, both in resource use and greenhouse gas emissions (Hasan and Soto, 2017). Growth rate and feed conversion efficiency are highly correlated, especially in fast growing genotypes compared to slower growing genotypes (Dunham, 2011).

Surveys indicate that consumers in China, the US and globally are likely to be more receptive to genetically engineered (GE) food if it lowers food costs or if the GE food is of enhanced nutritional quality (Curtis et al., 2004; Zhang et al., 2010). Thus, one major objective of the current study is to enhance the nutritional quality of catfish through genetic engineering by improving omega-3 fatty acid (FA) levels.

Omega -3 Fatty Acids

The long-chain omega-3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) have significant nutritional benefits in humans (Lauritzen et al. 2001). They are necessary for important biological processes of humans, such as lipid metabolism regulation, growth development stimulation, anticancer properties, anti-aging properties, immunoregulation, promoting cardiovascular health, aiding in weight loss, among others (Saunders et al., 2013). The American Dietetic Association and Dietitians of Canada recommend 500mg/day of EPA+DHA (Kris-Etherton et al., 2007). The long chain PUFAs are predominantly derived from marine fish. However, global capture of wild fish is currently in decline due to overfishing and environmental problems, leading to long chain PUFAs being less available.

Farmed fish could serve as an alternative source for EPA/DHA. However, most freshwater fish are unable to naturally synthesize long chain PUFAs, although they can convert the medium chain length into long chain ones to a greater or lesser extent depending on the species (Wang et al., 2014). This limits which aquaculture species provide the added health benefit of high levels of Ω-3 FAs (Wang et al. 2014). Catfish lack an efficient endogenous pathway for converting the short/medium chain fatty acids into long-chain PUFAs, such as EPA and DHA, and subsequently have Ω-3 FA levels 7-12 times less than Ω-3 rich fish such as salmon (Table 15; Wall et al., 2010).

Table 15. Mean total EPA and DHA content of fish and the amount of dietary fish required to provide approximately 1 gram of EPA and DHA per day.
This table is based on data from the USDA Nutrient Data Laboratory and the values can vary widely depending on species, season, diet, and packaging and cooking methods. Reprinted from Wall, R. et al., (2010)

Fish species	n-3 (EPA+DHA) content (g) per 100 g of fish	Amount of fish (g) required to provide 1 g EPA+DHA
Atlantic herring	2.01	50
Atlantic salmon	1.28–2.15	42.5–70.9
Sardines	1.15–2	50–87
Rainbow trout	1.15	87
Mackerel	0.4–1.85	54–250
Halibut	0.47–1.18	85–213
Tuna	0.28–1.51	66–357
Oyster	0.44	227
Flounder or Sole	0.4	204
Shrimp	0.32	313
Tuna (canned)	0.31	323
Cod	0.28	357
Haddock	0.24	417
Catfish	0.18	556
Scallop	0.2	500

Generation of fish lines capable of synthesizing EPA/DHA fatty acids from their feeds is a feasible solution. This might be achieved by overexpressing genes encoding enzymes involved in EPA/DHA biosynthesis. Fatty acid desaturases and elongases are among the key enzymes for the biosynthesis of PUFAs (Meesapyodsuk et al., 2007) (Figure 16). Among them, Δ6-, Δ5- and Δ4-desaturases and elongase are membrane-bound desaturases and fatty acid metabolic enzymes, which behave as important factors in EPA and DHA biosynthesis (Simopoulos, 2002). Δ5-desaturase uses eicosatetraenoic acid (ETA, 20:4n-3) as a substrate and allows the insertion of a double bond to produce EPA. The ETA fatty acid substrate is chain elongated (by elongases) from octadecatetraenoic acid (18:4n-3), a product of Δ6-desaturation of α-linolenic acid. Further, DHA is synthesized from EPA by the sequential chain elongation to docosapentaenoic acid (DPA, 22:5n-3) and then to 24:5n-3, followed by a Δ6-desaturation to 24:6n-3, which is finally retroconverted by peroxisomes to DHA (Sprecher, 2000). Alternatively, Δ4-desaturase can convert DPA directly into DHA. The insertion of a single transgene generally increased Ω-3 FA production 10-30%, sometimes as much as a 2X increase for some omega-3 fatty acids (but a simultaneous decrease for others).

Our lab has accomplished a high rate of targeted gene insertion. For example, 37% of fish integrated a masu salmon, *Oncorhynchus masou*, elongase transgene into exon 2 of luteinizing hormone (LH) gene of channel catfish (De et al., in preparation) using a modification of CRISPR/Cas9 termed 'Two-Hit by gRNA and Two-Oligos with a Targeting Plasmid' (2H2OP) (Yoshimi et al. 2016) with a great reduction in mosaicism. All 13 tissues tested across 3 individuals contained the transgene. Huang et al., (2021) generated multiple families of F1 β-actin-Δ 5

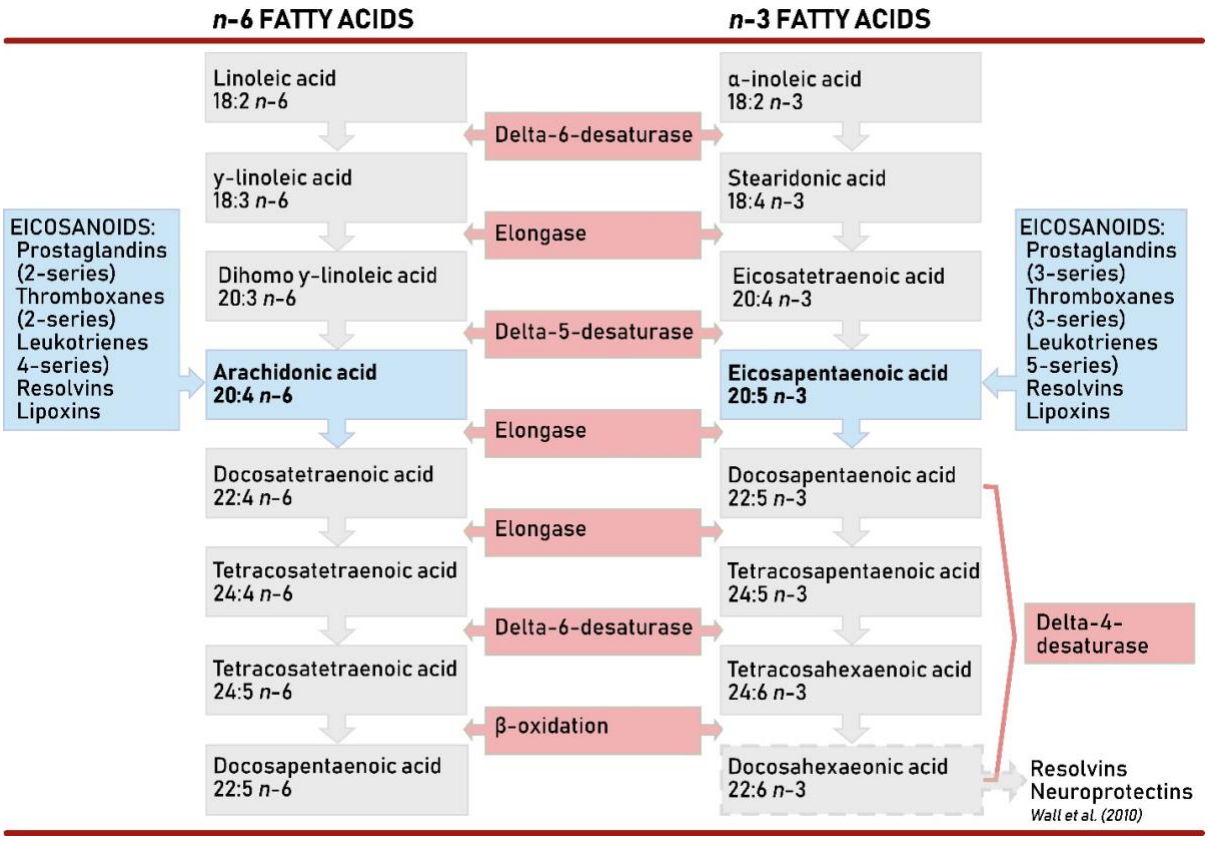


Figure 16. Pathway for Ω-3 FA and Ω-6 FA biosynthesis. Reprinted from Wall, R. et al., (2010)

transgenic channel catfish. 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. Levels of n-6 fatty acids, LA, AA, and DGA, decreased 1.11-fold in transgenic individuals. Individually, n-3 fatty acids ALA and DHA increased by 44.3% and 13.2% respectively. Precursors to n-6 delta5-desaturation, LA and DGA, decreased 13.2% and 11.9% respectively. Significant increases were seen on a mg/g weight basis for EPA, DHA, total HUFA.

Average coefficient of variation, 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 (Huang et al., 2021). Desaturase fish had an average variation in fatty acid levels of 7% while controls showed increased variation with a CV of 22%. 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.

MC4R

The central melanocortin pathway regulates energy homeostasis in vertebrates as well as somatic growth and feed efficiency. Natural mutations of MC4R in fish can affect growth. In the swordtail fish, *Xiphophorous nigrensis* and *X. multilineatus*, small and large male morphs point to a single locus encoding MC4R (Smith et al., 2015). Large male morphs in this species result from multiple copies of mutant forms of the receptor, at the *Y* chromosome-encoded P locus, that appear to function in a dominant negative fashion, blocking activity of the wild-type receptor.

Copy number variants of the MC4R gene have a dramatic effect on the onset of puberty in *Xiphophorus*, but in the closely related species, medaka, *Oryzias latipes*, MC4R had no effect on reproduction or puberty, and the knock-out of MC4R retarded embryonic development (Liu et al., 2019). In contrast, *in vitro* studies on the anadromous fish, spotted scat, *Scatophagus argus* revealed that MC4R regulates gonadotropin releasing hormone (GnRH) as well as follicle stimulating hormone (FSH) and luteinizing hormone (LH) both directly and indirectly (by affecting the expression of GnRH) (Jiang et al., 2017). These results indicate that mutations in the MC4R gene may lead to infertility.

The major objective of this study was to simultaneously knock out the MC4R gene while inserting masu salmon elongase (Evol2) transgene driven by the common carp β-actin promotor (ccβA-msEvol2) into channel catfish. After successful mutation, zygosity effects on growth rate was evaluated. Mutation rate, growth rate, survival, and gene expression at different target sites were compared. Finally, fatty acid levels in mutants and controls were determined and compared.

2. Materials and Methods

All experiments were conducted at the Fish Genetics Research Unit, E. W. Shell Fisheries Research Center, Auburn University, AL. All experimental protocols used in this experiment were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC) before the experiment was initiated and followed the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) protocols and guidelines.

2.1. Design and preparation of sgRNA and CRISPR/Cas9 System

A common carp β-actin promoter (Accession ID: M24113.1) was used to drive the expression of masu salmon elongase gene (Accesion ID: KC847063.1). An antifreeze polyA terminator from ocean pout, *Zoarces americanus* (Accession ID: S65567.1) was used to terminate transcription. This synthetic construct (ccβA-msEvol2), totaling 9,267bp, was built by GenScript (USA Inc., Piscataway, NJ 08854, USA). Two sets of two CRISPR short guide RNAs (sgRNA) plasmids were designed, targeting the coding region in exon one of the MC4R gene in channel catfish and the non-coding region adjacent to the PAM sequence of the transgene. The corresponding ccβA-msEvol2 transgene was designed to contain two arms, upstream and downstream, for homologous recombination, matching the cuts created by the sgRNA. sgRNAs and ssODNs were constructed for 2H2OP insertion of ccβA-msEvol2 into MC4R locus (Table 16). Each of the oligos and plasmid was reconstituted using DNase/RNase Free water to 10mM. To create template for sgRNA synthesis, the three oligos were used to were synthesize double stranded DNA (dsDNA) by mixing 25µl 2x EconoTaq Plus Master Mix (Lucigen), 12.5µl Universal Primer, and 12.5µl gene-specific oligonucleotide. PCR

Table 16. Primers used to amplify partial sequences of channel catfish, *Ictalurus punctatus*, oligonucleotides to target specified regions for CRISPR/Cas9 cleavage and single-stranded oligo donor nucleotide (ssODN). Universal primer was used to bind oligonucleotides to Cas-9 protein. Red letters indicate binding site to target gRNAs. MC4R-A, MC4R-B, MC4R-C, MC4R-A2-sense and MC4R-D-antisense were all used to target various loci in exon 1 of the channel catfish MC4R gene. MSTN-1, MSTN-2 and MSTN-3 were all used to target various loci in exon 1 of the channel catfish MC4R gene. MC4R-ALL-1F and MC4R-ALL-1R were used to amplify DNA segments flanking the target sites for guide RNAs in the catfish MC4R gene. EX1-F and EX1-R were used to amplify DNA segments flanking the target sites for guide RNAs in the catfish MC4R gene. PUC57 Vector was used to amplify a partial sequence of the ccβA-msEvol2 plasmid containing the masu salmon, *Onchorhynchus masou*, elongase gene driven by the common carp β-actin promoter. BsalElvol2_ ssODN1 and BsalElvol2_ ssODN2 were used to flank the cut site associated with the MC4R-A2-sense gRNA to facilitate homologous directed repair (HDR) in the MC4R gene. BsalElvol2_ ssODN3 and BsalElvol2_ ssODN4 were used to flank the cut site associated with the MC4R-D-antisense gRNA to facilitate homologous directed repair (HDR) in the MC4R gene.

Oligo sequence (5' to 3')	Oligo name
AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	Universal Primer
taatacgactcactataGGGATGGCGCTGATCACCAGgttttagagctagaa	MC4R-A
taatacgactcactataGGGAAAGGAACTCGGAGTCgttttagagctagaa	MC4R-B
taatacgactcactataGGGCAGGATGGTGAGCGTCAgttttagagctagaa	MC4R-C
taatacgactcactataGGTGGTGGCGTTCGGTCCGAgttttagagctagaa	MSTN1
taatacgactcactataGGGCGAGGCGCAGTGTTCAGgttttagagctagaa	MSTN2
taatacgactcactataGGGTCGAGCAGCTGCTGCACgttttagagctagaa	MSTN3
GGAGATGGAGGACACGGAAG	MC4R-F
GAGACATGAAGCAGACGCAATA	MC4R-R
taatacgactcactataGTGATGGCGCTGATCACCAGgttttagagctagaa	MC4R-A2-sense
taatacgactcactataCGGGATGCAGCATGCACACCgttttagagctagaa	MC4R-D-antisense
taatacgactcactataCTTGTCTGTAAGCGGATGCCgttttagagctagaa	PUC57 Vector
TATCGAACGCGACAGAAACGGCTGTGATGGCGCTGATCACGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTG	BsalElvol2_ ssODN1
GCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATCAGCGGCAACCTGACCATCTCTGGAGACGTCGTGAAAAGC	BsalElvol2_ ssODN2
GAGGAGGTCTTGCGGATATGAACGTGTCGGAGCACCACGGGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTG	BsalElvol2_ ssODN3
GCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGATGCAGCATGCACACCGGAACCACAGCCTGGGCGTGCAG	BsalElvol2_ ssODN4
ACTCCTCTCTGAGACCTGAC	EX1-F
AGTTAGGCTATGAAGCAGTAGT	EX1-R
CGAAATCCGTTCCTTTTTACTG	Evol2-F
CTGGCCTGTTCCTCATGTATTT	Evol2-R

cycling was carried out with initial denaturing at 95°C for 3 min; 5 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec with a ramp speed of -0.2°C/sec, extension at 72°C for 30 sec; 20 cycles of denaturation at 95°C for 30 sec, extension at 72°C for 20 sec with a ramp speed of -0.2°C/sec; and final extension at 72°C for 10 min.

The Taq Polymerase was then inactivated using 4.8µl of 0.5M EDTA and incubated at 75°C for 20 min. The PCR product was confirmed using 1% agarose gel. The sgRNA was synthesized using the Maxiscript T7 Kit (Thermo Fisher Scientific), following the manufacture guidelines. A mix of 2µl dH2O, 10µl dsDNA template (created by PCR as described above), 2µl 10X buffer, 1µl ATP, 1µl CTP, 1µl GTP, 1µl UTP and 2µl Enzyme Mix was incubated at 37°C for 90 min. The solution was cleared of DNA contamination by adding 1µl of Turbo DNase I, vortexing briefly and incubating at 37°C for 15 min. The magnesium ions were chelated by adding 5µl of 0.5M EDTA. The Turbo DNase I was inactivated by heating the solution at 75°C for 10 min. The obtained sgRNAs were purified using Zymo RNA Clean and Concentrator kit (Zymo Research). The sgRNAs were stored in -80°C freezer.

The Cas9 protein was acquired from PNA Bio (3541 Old Conejo Rd, Newbury Park, CA 91320) and reconstituted in dH_20 to a concentration of 1mg/ml. The ccβA-msEvol2 plasmid was reconstituted to 250ng/µl. Each of the ssODNs were reconstituted to 100ng/µl. Twenty minutes prior to fertilizing the eggs, two sets of injection solutions were prepared by mixing 2µl (200ng) of each pair of ssODN, 1 µl ccβA-msEvol2 plasmid (125ng), 1µl ccβA-msEvol2 sgRNA (300ng), 1µl MC4R-(A or D) sgRNA (300ng), 2 µl Cas9 and 2 µl phenol red (10%) to a total volume of 11 µl.

2.2. Brood stock husbandry, selection and spawning

Brood stock were cultured in 0.04-ha earthen ponds averaging 1-meter in depth. They were fed a 32 percent protein catfish pellet at 1-2% of their body weight five days per week. Dissolved oxygen was maintained above 3 mg/L using a ½ horsepower surface aerator (Air-O-Lator).

The Kansas strain of channel catfish was chosen as broodstock due to their superior growth and fry output when induced by injection of luteinizing hormone releasing hormone analogs (LHRHa). Individuals were chosen based on their health and secondary sexual characteristics. Males with well-developed papilla and large, muscular heads that were wider than the rest of their bodies were chosen. Dark coloring and scarring from territorial fighting were also signs of quality males in reproductive condition. Females with soft, well-rounded abdomen that were wider than their head, and a swollen urogenital opening were chosen. Broodstock were minimally handled and kept in tanks for as short a time as possible to reduce stress.

Males were terminated by a percussive blow to the head followed by pithing. The body cavity was opened carefully with a scalpel, ensuring not to pierce any organs. Testes were removed using tweezers and/or scissors and washed in a weigh boat with 0.9 percent saline using bottled distilled water, removing any blood or tissue. Excess water was drained and the testes weighed before macerating the testes using scissors to release sperm. The homogenized testes were then filtered into a 50mL Falcon tube using a 100-micron screen. The sperm was then diluted with 0.9 percent saline solution to a maximum of 10mL/gram of testes. Sperm concentration was tested using a Nanodrop 2000 Spectrophotometer (Thermo Scientific) and

running a simple linear regression between sperm concentration and absorbance using the equation y=-2.450+0.185lnX (Adjusted R2=0.601) at 450nm (Cuevas-Uribe & Tiersch, 2011). Motility was analyzed under a 10X light microscope while sperm count was checked under 40X magnification. Ideally, sperm was used immediately, but could be stored at 4°C for one week, with declining quality or up to two weeks with the addition of gentamycin at a concentration of 1mg/mL sperm solution.

Females were weighed and gently placed in labeled 32mm mesh bags. The spawning bags were then placed in a flow through holding tank so that the fish was fully submerged, ensuring that water quality was ideal with dissolved oxygen levels above 5 mg/L. A 14-gauge implanter was loaded with 100µg/kg body weight of luteinizing hormone releasing hormone analog (LHRHa) implant. The needle was then inserted at a 45-degree angle ventrally adjacent to the pelvic fin and the implant was inserted. The ovulation time was predicted based on the degree-hours according to Phelps et al. (2007). Water temperature ranged from 26-28°C. Females were checked 36 hours after LHRHa injection and every four hours after that until ovulation. Once a female began ovulating, indicated by eggs visible on the bag, she was carefully transferred into a tank with 100 mg/L buffered tricaine methane sulfonate (MS-222) with equal parts sodium bicarbonate until completely anesthetized. Prior to this time, a thin layer of vegetable shortening, Crisco, was used to coat several 20cm diameter metal egg collection pans. The fish was then dipped in fresh water to wash off the MS-222 and gently dried with a towel. A thick layer of vegetable shortening was applied around the urogenital opening, including the pelvic fins to prevent sticking of the eggs during stripping. The female was hand stripped into the greased pan by gently applying pressure on the abdomen from the anterior end of the abdomen posteriorly towards the vent. Eggs would

usually flow freely, well rounded and golden in color, with minimal blood. The stripping ensured that the eggs could not contact freshwater, which can activate the eggs. Eggs were then covered with another spawning pan to maintain moisture and transported to the molecular laboratory.

Approximately 200-300 eggs were transferred to a greased pan for fertilization. Approximately 3mL of sperm solution was added to the eggs and mixed gently with fingertips. Fresh water was added to barely cover the eggs in order to activate the sperm and eggs and the water was swirled to form a single layer and prevent sticking. After two minutes, the eggs should be fertilized, and three more cm of water was added to the eggs and the eggs were left to harden for 15 minutes.

While the embryos were hardening, 5-10µl of the Cas9/sgRNA/phenol red mixture was loaded into 1.0mm OD borosilicate glass capillary microinjection needles using a microloader. The tips of the needles were opened by breaking the end with a scalpel. The needle was then inserted into the micropipette holder to its deepest range and tightened, ensuring a tight seal. The compressed air cylinder was opened, and pressure was adjusted to 7000kPa and 0.824 m^3/hr using the pressure regulator. The injection volume was adjusted to 50nl by manipulating the pressure, the length of injection and the needle diameter. Injection volume was measured by injecting a drop of mineral oil on a hemocytometer.

After 15 minutes 100-200 embryos were transferred in a single layer to a greased 100mm petri dish and covered with Holtfreter's solution (Table 17). The petri dish was placed on the stage

Table 17. Name, type of material, company, catalog number (if applicable) of materials and equipment used in microinjection protocol for CRISPR/Cas9 in channel catfish, *Ictalurus punctatus*.

Name of Material/ Equipment	Type	Company	Catalog Number	Comments/Description
Reproboost® implant	Hormone	Center of Marine Biotechnology		Luteinizing hormone releasing hormone analog (LHRHa) for artificial spawning
TRICAINE-S	Anesthesia	Western Chemical. Inc.		For sedation of brood stock fish during hormone injection and egg stripping.
Phenol red	Reagent	Sigma-Aldrich	P0290	0.5%, sterile filtered
Stereo microscope	Equipment	Olympus	213709	For visualizing the eggs during microinjection
Microinjector	Equipment	ASI-Applied Scientific Instrumentation	Model MPPI-3	For the delivery of the injection material into the embryos
Micromanipulator	Equipment	ASI-Applied Scientific Instrumentation	Model MM33	For holding and controlling the movement of the injection needle.
Eppendorf Microloader	Tool	Eppendorf	5242956.003	For loading injection solution into microinjection needles.
Vertical needle puller	Equipment	David Kopf Instruments	Model 720	For pulling microinjection needles
Borosilicate glass capillaries	Tool	Fisher Scientific		1 mm outer diameter (OD), for making microinjection needles.
Petri dish	Tool	VWR	25384-302	For holding the embryos during the microinjection.
Crisco®	Vegetable shortening	The J.M. Smucker Company		For coating spawning pans and petri dishes.
Holtfreter`s solution	Reagent	Lab Made		59 mM NaCl, 0.67 mM KCl, 2.4 mM $NaHCO_3$, 0.76 mM $CaCl_2$, 1.67 mM $MgSO_4$ (Armstrong et al., 1989) to incubate the microinjected embryos till hatch.
Doxycycline hyclate USP (monohydrate)	Antibiotic	Letco Medical	690904	Added to Holtfreter's solution to 10 ppm to prevent bacterial infections.

of the microscope. In one smooth motion, the needle was lowered until it pierced the chorion and yolk, and the pedal of the microinjector was depressed, delivering the CRISPR solution, and withdrawing slowly. For best results, the solution was injected as close to the blastodisc as possible. When the blastodisc was not visible, the solution was spread throughout the embryo by depressing the pedal while simultaneously withdrawing the needle smoothly. To reduce mosaicism, embryos were injected between 15 min and 90 min post-fertilization, while they remained in the one cell stage. Injection controls were injected with 50nl of phenol red, while non-inject controls were not injected.

Embryos were placed in four-liter tubs of Holtfreter's solution with 10 mg/L doxycycline kept at 27°C with continuous aeration. The solution was changed, and dead embryos were removed daily. After about 5 days, or when the embryos were moving rapidly within the egg membrane and close to hatch, doxycycline treatment was discontinued. At 20 dph fry were moved to aquaria in recirculating systems until large enough to be PIT (Passive Integrated Transponders) tagged and moved to earthen ponds.

2.3. Mutation Analysis

Pelvic fin-clip samples (10-20mg) were collected in sterile 1.5mL Eppendorf tubes and kept in a -80°C freezer until DNA extraction. Genomic DNA was extracted using proteinase K digestion and ethanol precipitation using the following protocol: fin clips were digested in 600µl of cell lysis buffer (100mM NaCl, 10mM Tris, 25mM EDTA, 0.5% SDS) and 2.5µl of proteinase K in a 55°C hot water bath for 4-8 hours, with occasional vortexing. Protein was precipitated by adding 200µl of protein precipitation solution (Qiagen, 19300 Germantown Road Germantown,

MD 20874), vortexed, stored on ice for 12 minutes and centrifuged for 8 minutes at 15,000rcf. The supernatant containing DNA was then precipitated with isopropanol followed by centrifugation for 5 minutes at 15,000rcf and finally washed twice with 75% ethanol by inverting gently 5-times before being dissolved in dH_2O. DNA concentration and purity was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and concentration was adjusted to 500ng/µl.

To determine the presence of the transgene, the primer set Evol2-F, Evol2-R (Table 15) was designed using Primer3plus to encapsulate all possible mutation sites in the ccβA-msEvol2 plasmid. The Expand High Fidelity^{PLUS} PCR System (Roche) was used with 500ng of genomic DNA. A Bio-Rad T100 Thermal Cycler was used to run PCR with an initial denaturing at 95°C for 3 min; 34 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 40 sec with a ramp speed of -0.2°C/sec, extension at 72°C for 40 sec; and final extension at 72°C for 10 min. The PCR product was confirmed on a 1% TAE Tris base, acetic acid and EDTA agarose gel. The knock-in mutation was preliminarily identified by presence of the amplified DNA fragment.

The primer set MC4R-F, MC4R-R (Table 15) was designed using Primer3plus to encapsulate all possible mutation sites in the MC4R gene. The Expand High Fidelity^{PLUS} PCR System (Roche) was used with 500ng of genomic DNA. A Bio-Rad T100 Thermal Cycler was used to run PCR with an initial denaturing at 95°C for 3 min; 34 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 40 sec with a ramp speed of -0.2°C/sec, extension at 72°C for 40 sec; and final extension at 72°C for 10 min.

The PCR product was confirmed on a 1% TAE Tris base, acetic acid and EDTA agarose gel. The PCR product from the treatment fish was mixed with PCR product from a wild type control of the same family at a 1:1 ratio. The combined product was then hybridized in a BioRad Thermocycler using an initial denaturing at 95°C for 3 min; 85°C for 1 min with a ramp speed of -0.2°C/sec, 75°C for 1 min with a ramp speed of -0.2°C/sec, 65°C for 1 min with a ramp speed of -0.2°C/sec, 55°C for 1 min with a ramp speed of -0.2°C/sec, 45°C for 1 min with a ramp speed of -0.2°C/sec, 35°C for 1 min with a ramp speed of -0.2°C/sec, 25°C for 1 min with a ramp speed of -0.2°C/sec.

The Surveyor® mutation detection kit (Integrated DNA Technologies) was used to detect mutations. Hybridized products were mixed with Nuclease S, Enhancer S, MgCl2 and Reaction Buffer (2) according to kit instructions and incubated at 42°C for one hour. The digested products were separated on a 1.5 percent TBE (Tris borate EDTA) agarose gel and compared with that of control samples.

To confirm and identify the mutations, positive samples were sequenced using the TA cloning method. The largest individuals from each treatment that repeatedly were confirmed as mutants using Surveyor Analysis (Fisher Scientific) were sequenced. First, genomic DNA from three mutants per treatment was amplified with PCR using Expand High Fidelity^{PLUS} PCR System (Roche) using the above protocol. The PCR product was verified using a 1 percent TAE agarose gel and cloned into the TOPO® TA Cloning® Kit (Invitrogen) with 20 clones per sample and sent to MCLabs for sequencing. Finally, the resulting sequences were interpreted using the MAFFT sequence alignment tool.

2.4 Fatty Acid Analysis

Muscle was sampled from 3 fish containing the ccβA-msEvol2 plasmid and mutated at the MC4R gene, 3 fish containing the MC4R mutation and not the ccβA-msEvol2 plasmid and 3 control fish with no mutation. Muscle samples were taken immediately after euthanizing the individual. They were then prepared for lipid extraction by first grinding into a slurry with a coffee grinder. Two grams of ground tissue was homogenized per extraction using a handheld tissue homogenizer until tissue was sufficiently homogenized. Lipid extraction was performed using the chloroform-methanol protocol from Folch et al. (1957) and done in triplicate for each sample. Extracts in hexane were kept in 2 ml borosilicate glass vials with PTFE caps and stored at -20°C until they could be injected into a gas chromatograph (GC-MS7890A). The concentration of the fatty acid was measured using gas chromatography-mass spectrometry (GC-MS, Agilent Technologies 7890A GC with 5975C MS) equipped with an DB-1701 GC column. Helium was used as the carrier gas. The initial temperature of the oven was set at 100 °C for 5 min, followed with a ramp of 4 °C/min to reach 250 °C and then held for 10 min. The detector was kept at 225 °C. Peaks were identified and quality check of the resulting spectra, and calculation of concentrations and was done by comparing sample retention times to a standard mix (Supelco® C4-24 Fatty Acid Methyl Ester (FAME) Mix; Lot: LRAC7954).

2.5 Culture and growth

Fry from each genetic type were stocked into 3-replicate 50L aquaria in recirculating systems for growth experiments. Fish were kept at a density of 1 fish/L. Fish in each aquarium were fed ad-libitum with Aquamax powdered and pelleted fish diets and catfish diets with the

appropriate fatty acid content. Based on our previous results with transgenic desaturase common carp (Cheng et al., 2014), the diets needed adequate precursors, Ω-6 fatty acids, for the desaturase transgenic catfish to produce Ω-omega-3 fatty acids in the muscle. In fact, diets high in Ω-3 fatty acids did not result in more Ω-3 fatty acids in the muscle of desaturase transgenics, but diets high in Ω-6 fatty acids did allow the desaturase transgenic carp to produce elevated Ω-3 fatty acids.

Feed size was adjusted as the fish grew. Fry were fed Purina® AquaMax® powdered starter feed until they were large enough to eat Purina® AquaMax® 100. Both feeds contained 50% protein. All fish were fed every day to satiation. The fish were sampled at 6-months post hatch.

2.8. Statistical analysis

To calculate differences in body weight between ccβA-msEvol2 knockout/MC4R knock in mutants, MC4R knockout mutants and controls, a one-way ANOVA and Tukey's multiple comparisons test were performed using R programming language (R Core Team, Vienna, Austria). In cases where different treatments were kept in separate aquaria at varying densities, a regression based on density was calculated, and weights were adjusted accordingly before running the statistical analysis. Differences in mutation rate were calculated with logistic regression using R programming language.

3. Results

3.1. Growth

A total of 19 P1 Elongase transgenic MC4R knock-out channel catfish (ccβA-msEvol2), 33 Non-inject control (NIC) and 31 Inject-control (Inj-Cntrl) were generated in 2019. CcβA-msEvol2 mutants were 56% and 14.59g larger than NIC fish (p=0.001) at 6-months post hatch (Figure 17; Table 18). ccβA-msEvol2 mutants were 29% and 9.27g larger than Inj-Cntrl fish (p=0.056). At 6-months post hatch, ccβA-msEvol2 mutants were 41.81% and 12.01g larger than controls (p=0.005).

3.2. Mutation Rate

A total of 53 fish survived microninjection of CRISPR/Cas9, ccβA-msEvol2 transgene and the MC4R-D sgRNA. A total of 13/53 (25%) had a mutation in the MC4R target site and 3/53 (6%) integrated the ccβA-msEvol2 plasmid (Table 19). None of the 53 MC4R-D fish had both a mutation in the MC4R gene and insertion of the ccβA-msEvol2 transgene. A total of 39 fish survived microninjection of CRISPR/Cas9, ccβA-msEvol2 transgene and the MC4R-A sgRNA. A total of 36/39 (92%) fish had a mutation in the MC4R target site and 21/39 (54%) integrated the ccβA-msEvol2 plasmid at the MC4R target site. Nearly half (49%) of the 39 MC4R-A fish had both a mutation in the MC4R gene and insertion of the ccβA-msEvol2 transgene. A single band observed using the Evol2 primer set indicates integration of the ccβA-msEvol2 transgene (Figure 18). Multiple bands using the MC4R primer set corresponded to expected cut sites in MC4R gene (Figure 19). Each positive result was confirmed with a second gel. MC4R-A generated a greater rate of knockout of MC4R (p=1.14e-7) and knock-in of ccβA-msEvol2 (p=1.12e-5) than MC4R-D. Sequencing indicates insertion with complete fidelity into the channel catfish genome.

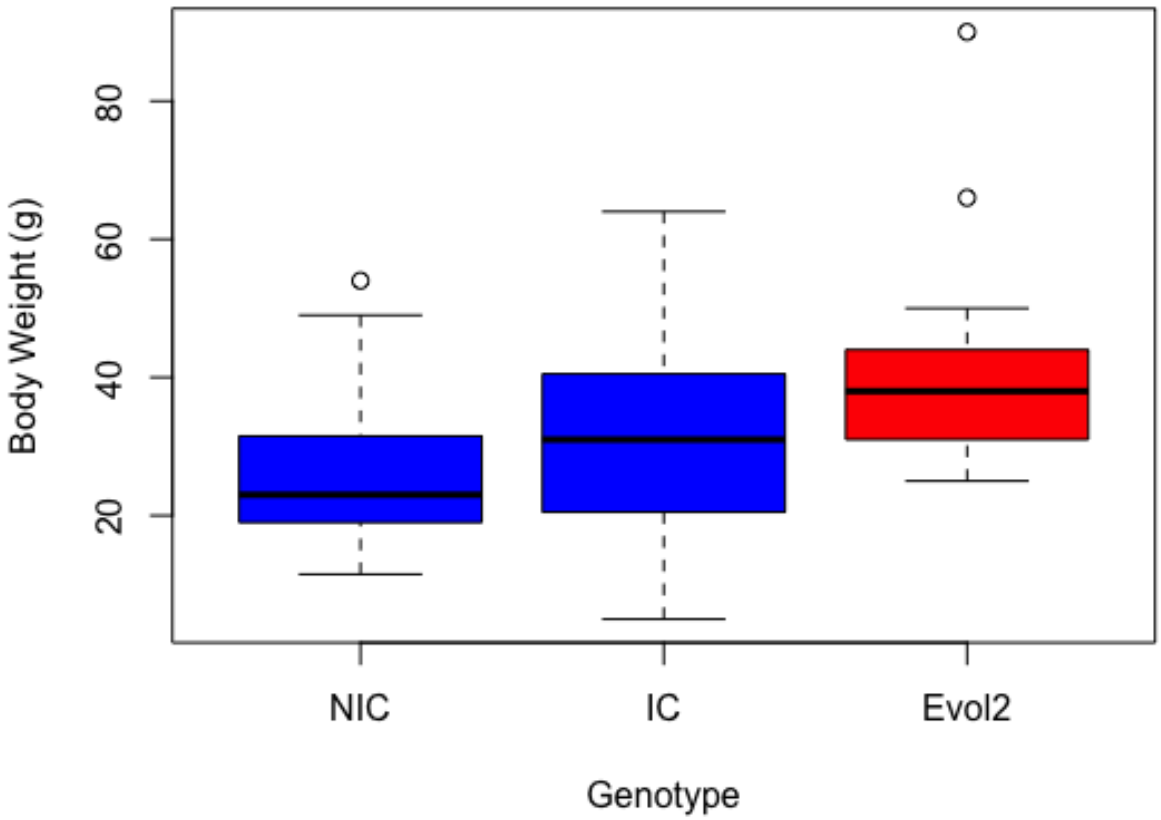


Figure 17. Box and whisker plot of body weights (grams) of Non-Inject Control (NIC; blue), Inject-Control (IC; blue) and P1 ccβA-msEvol2 transgenic MC4R knock-out (Evol2; red) channel catfish, *Ictalurus punctatus*, generated in 2020, at 6-months post hatch. The ccβA-msEvol2 transgene containing masu salmon, *Oncorhynchus masou*, elongase gene driven by the common carp, *Cyprinus carpio*, beta-actin promoter was inserted into the channel catfish melanocortin-4 receptor (MC4R) gene using the 2-hit 2-oligo with plasmid (2H2OP) method. Mutants and controls were kept in separate 50L aquaria at a maximum density of 1 fish/L and fed daily to satiation. The plot shows median of the data (dark line), the lower and upper quartiles (25% and 75%; top and bottom of the box respectively), the area 1.5 times the interquartile range (whiskers), and outlier (circle). At 6-months post hatch, ccβA-msEvol2 mutants were 41.81% and 12.01g larger than controls (p=0.005).

Table 18. Mean weights (grams), sample size (N), standard deviation (SD) and coefficient of variation (CV) of Non-Inject Control (NIC), Inject-Control (Inj-Cntrl), overall controls (CNTRL) and P1 ccβA-msEvol2 transgenic MC4R knock-out (Evol2) channel catfish, *Ictalurus punctatus*, at 6-months post hatch. The ccβA-msEvol2 transgene containing masu salmon, *Oncorhynchus masou*, elongase gene driven by the common carp, *Cyprinus carpio*, beta-actin promoter was inserted into the channel catfish melanocortin-4 receptor (MC4R) gene using the 2-hit 2-oligo with plasmid (2H2OP) method. Mutants and controls were kept in separate 50L aquaria at a maximum density of 1 fish/L and fed daily to satiation. Overall, at 6-months post hatch, ccβA-msEvol2 mutants were 41.81% and 12.01g larger than controls (p=0.005). A paired t-test was used to compare treatments.

Mean Body Weight (g) at 6-months post hatch

	N	Mean	SD	CV
NIC	33	26.15	10.74	41.05
IC	31	31.47	15.04	47.81
Evol2	19	40.74	15.52	38.10
CNTRL	64	28.73	13.17	45.85
Evol2	19	40.74	15.52	38.10

Table 19. Mutation rates of two gRNA (MC4R-D and MC4R-A; Table 1) targeting different loci in exon one of the channel catfish, *Ictalurus punctatus*, for CRISPR/Cas9 cleavage and simultaneous insertion of the ccβA-msEvol2 plasmid into the cut site. P1 fish were generated in 2020. The ccβA-msEvol2 transgene containing masu salmon, *Oncorhynchus masou*, elongase gene driven by the common carp, *Cyprinus carpio,* beta-actin promoter was inserted into the channel catfish melanocortin-4 receptor (MC4R) gene using the 2-hit 2-oligo with plasmid (2H2OP) method. Approximately 50nl of solution, composed of 1µg/µl Cas9 protein (1µl), 400ng/µl sgRNA1 (0.5µl), 400ng/µl sgRNA2 (0.5µl), 50ng/µl donor plasmid (1µl), 100ng/µl ssODN1 (0.5µl), 100ng/µl ssODN2 (0.5µl) and 60% phenol red (1µl), was injected into each embryo close to the blastodisc 15 min after fertilization using a glass capillary needle. Knockout and knock-in are denoted by KO and KI, respectively. Logistic regression was used to determine significant difference in mutation rates between MC4R-D and MC4R-A.

	MC4R KO Mutation Rate	ccβA-msEvol2 KI Mutation Rate	Both	Homozygosity KO
MC4R-D	25% (13/53)	6% (3/53)	0% (0/53)	38% (5/13)
MC4R-A	92% (36/39)	54% (21/39)	49% (19/39)	69% (25/36)
p-value	1.14e-7	1.12E-05		0.056

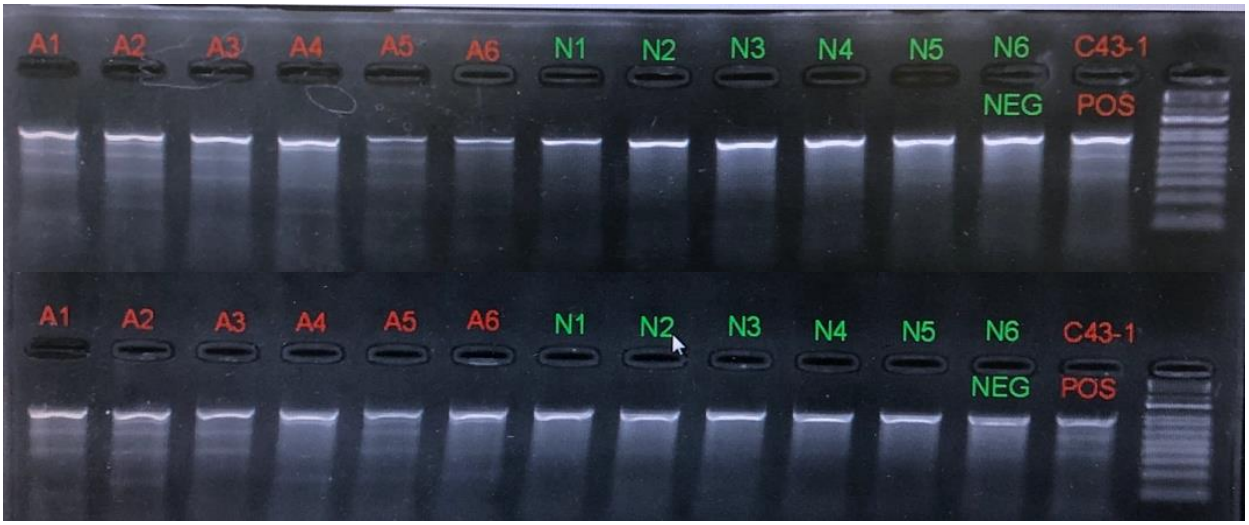


Figure 18. The ccβA-msEvol2 plasmid containing masu salmon, *Oncorhynchus masou*, elongase gene driven by the common carp, *Cyprinus carpio,* beta-actin promoter was inserted into the channel catfish melanocortin-4 receptor (MC4R) gene using the 2-hit 2-oligo with plasmid (2H2OP) method. Six (A1-A6) channel catfish, *Ictalurus punctatus*, were microinjected with MC4R-A sgRNA in conjunction with ccβA-msEvol2 plasmid (Table 15). P1 fish were generated in 2020. Approximately 50nl of solution, composed of 1µg/µl Cas9 protein (1µl), 400ng/µl sgRNA MC4R-A (0.5µl), 400ng/µl sgRNA2 (0.5µl), 50ng/µl donor plasmid (1µl), 100ng/µl ssODN1 (0.5µl), 100ng/µl ssODN2 (0.5µl) and 60% phenol red (1µl), was injected into each embryo close to the blastodisc 15 min after fertilization using a glass capillary needle. All six fish tested using Surveyor mutation detection kit were mutants. Six channel catfish (N1-N6) were not injected to use as controls. None of the controls showed a mutation in the MC4R gene. Sample C43-1 was previously identified as an MC4R mutant and was used as a positive control. The top row was hybridized with wild-type by mixing equal volumes of sample PCR product with N6 negative control in order to identify homozygotes. The bottom row was not hybridized with wild-type in order to identify heterozygotes.

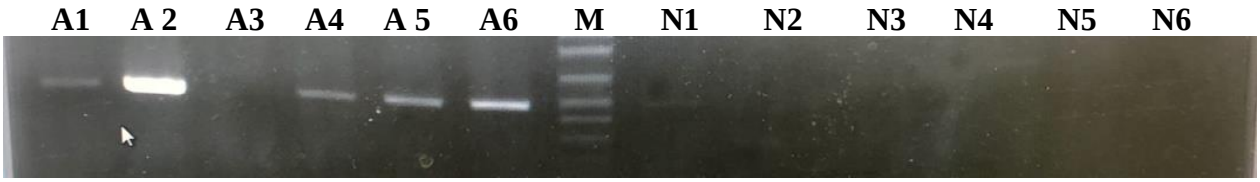


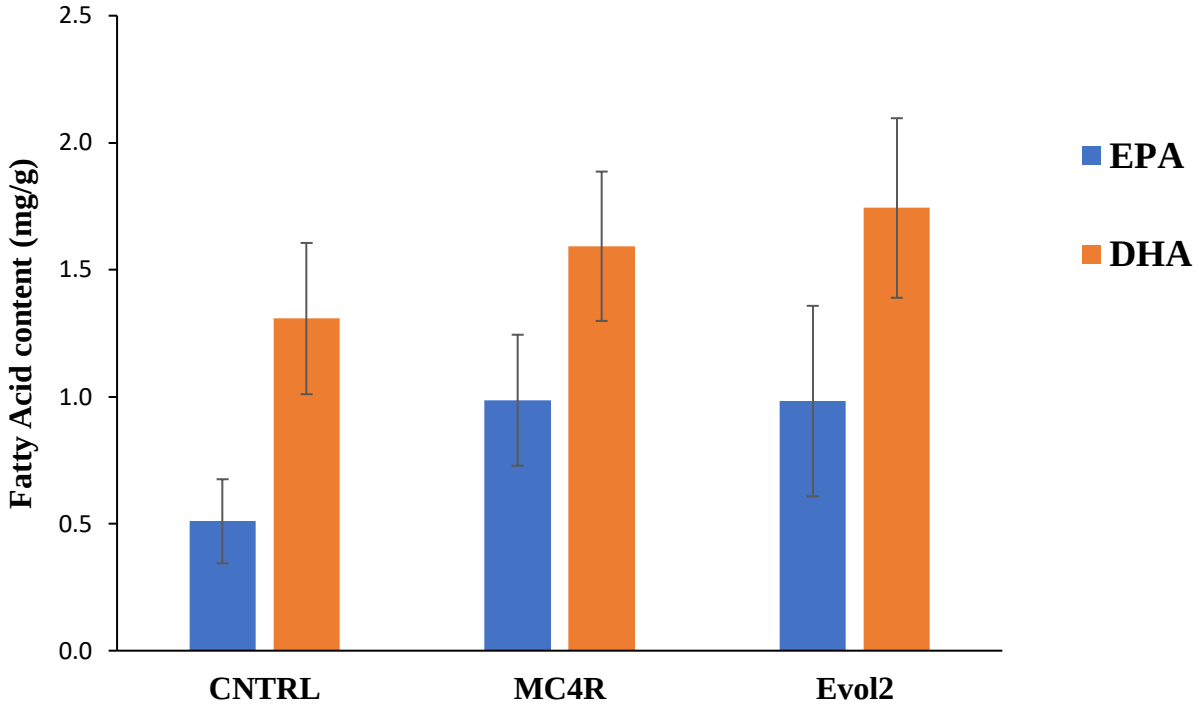
Figure 19. The ccβA-msEvol2 plasmid containing masu salmon, *Oncorhynchus masou*, elongase gene driven by the common carp, *Cyprinus carpio*, beta-actin promoter was inserted into the channel catfish melanocortin-4 receptor (MC4R) gene using the 2-hit 2-oligo with plasmid (2H2OP) method. Six (A1-A6) channel catfish, *Ictalurus punctatus*, were microinjected with MC4R-A sgRNA in conjunction with ccβA-msEvol2 plasmid (Table 15). P1 fish were generated in 2020. Approximately 50nl of solution, composed of 1µg/µl Cas9 protein (1µl), 400ng/µl sgRNA MC4R-A (0.5µl), 400ng/µl sgRNA2 (0.5µl), 50ng/µl donor plasmid (1µl), 100ng/µl ssODN1 (0.5µl), 100ng/µl ssODN2 (0.5µl) and 60% phenol red (1µl), was injected into each embryo close to the blastodisc 15 min after fertilization using a glass capillary needle. Six channel catfish (N1-N6) were not injected to use as controls. Five out of six channel catfish (A1, A2, A4, A5, A6) are positive for the ccβA-msEvol2 transgene as indicated by 485bp band. Six channel catfish (N7-N12) served as non-inject controls. None of the controls tested positive for the transgene. M indicates 1kb marker.

3.3. Fatty Acid Levels

There were no statistically significant differences in EPA ($p=0.203$) and DHA ($p=0.380$) levels between any of the groups ($p=0.203$) (Table 20). However, observed mean EPA levels in MC4R mutants and ccβA-msEvol2/MC4R mutants were 94.12% and 92.16% higher than controls, respectively (Figure 20). Mean observed DHA levels for MC4R mutants and ccβA-msEvol2 /MC4R mutants were 21.37% and 32.82% higher than controls, respectively (Figure 23). Overall observed, levels of EPA+DHA were 50.00% and 5.72% higher in ccβA-msEvol2/MC4R fish than controls and MC4R fish.

Table 20. Mean eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) content (mg) per gram of muscle tissue in control (CNTRL), melanocortin-4 receptor (MC4R) knockout and MC4R knockout + ccβA-msEvol2 transgene (Evol2) knock-in channel catfish, *Ictalurus punctatus*. The ccβA-msEvol2 transgene containing masu salmon, *Oncorhynchus masou*, elongase gene driven by the common carp, *Cyprinus carpio*, beta-actin promoter was inserted into the channel catfish melanocortin-4 receptor (MC4R) gene using the 2-hit 2-oligo with plasmid (2H2OP) method. Muscle was sampled from 3 fish from each treatment. There was no significant difference in EPA or DHA levels between any of the groups using ANOVA (p=0.203; p=0.380).

	Mean Fatty Acid Content in Muscle (mg/g)										
	EPA				**DHA**				**EPA+DHA**		**Amount of fish (g) required**
	N	Mean	SD	CV	Range	Mean	SD	CV	Range	Mean	Range	**to provide 500mg EPA+DHA**
CNTRL	3	0.51	0.33	64.92	0.14-0.72	1.31	0.60	45.55	1.16-1.69	1.82	1.62-1.88	275.17
MC4R	3	0.99	0.52	52.32	0.93-1.06	1.59	0.59	36.93	1.29-2.15	2.58	2.35-3.09	193.94
Evol2	3	0.98	0.75	76.35	0.55-2.34	1.74	0.71	40.56	1.39-1.95	2.73	2.35-4.23	183.45



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4. Discussion

In the current study, the effects of microinjection of sgRNA targeting exon 1 of the channel catfish melanocortin-4 receptor gene in conjunction with ccβA-msEvol2 elongase plasmid, ssODNs and Cas9 protein on mutation rate, growth and omega-3 content were investigated. Efficient mutagenesis was achieved. There was high variability in mutation rate between both target sites with 49% of fish microinjected with MC4R-A having both the elongase insertion and MC4R deletion compared to 0% of MC4R-D fish. Microinjection of MC4R-A produced a mutation rate in the MC4R gene of 92%. This is far higher than the 33% mutation rate achieved in our lab in 2017 using the same sgRNAs (Coogan, This dissertation, Chapter 3). The two major differences in protocol between this study and previous experiments are the 2H2OP method and simultaneous insertion of the ccβA-msEvol2 transgene. The previous experiment used CRISPR/Cas9 with sgRNAs specifically designed to knockout the MC4R gene without adding in a transgene. Growth and overall omega-3 content was 45% and 50% higher, respectively, in MC4R/ccβA-msEvol2 mutants than in controls.

In regard to mutation rate, Yoshimi et al., (2016) reported knockout mutations of 82.4% for the Rosa26 locus and integration of the green fluorescent protein (GFP) of 17.6% in rats, *Rattus norvegicus*. The 2H2OP method, designed by Yoshimi et al. (2016), uses ssODNs as donor templates to facilitate homologous recombination (HR) rather than non-homologous end joining (NHEJ) to repair the double stranded break (DSB). NHEJ is the faster and typically more favored repair mechanism and is far more mutagenic, making it desirable for generating knockouts Mao et al., 2008. However, by introducing a plasmid with ssODNs homologous to the cut site, the gap can quickly be filled with the plasmid, thereby preventing reconstitution of the original sequence

(Yoshimi et al., 2016). Additionally, the 5' degradation of ssODNs by exonucleases and removal by helicase in the 2H2OP method can lead to incomplete repair and cause a high rate of indel mutations (Yoshimi et al., 2016). The insertion of an entire transgene virtually guarantees complete knockout of the target gene and could cause greater phenotypic changes than smaller indels.

Growth was 45% higher in MC4R/ccβA-msEvol2 mutants than in controls. Previous MC4R knockout channel catfish generated in our lab in 2017 were 18.87% larger than controls at a similar size (~50g) to this study (Michael Coogan, this dissertation, Chapter 3) and 56% and 37% larger than controls at 600g and 3kg, respectively, in initial aquaria experiments in 2016 (Dunham, personal communication). The much larger gains achieved with MC4R/ccβA-msEvol2 at the small fingerling stage could be due to pleiotropic effects of the transgene, a greater truncation of the MC4R protein, leading to greater phenotypic gain, or family effects. This faster growth makes MC4R/ccβA-msEvol2 mutants a potentially high value genotype for commercial aquaculture. While these results are promising, the experiment was not performed under commercial settings and future research should evaluate whether there is a genotype x age or genotype x environment interaction. Additionally, as these fish were P1 generation and were very likely mosaics, future research should evaluate performance in the F1 generation and the role of zygosity in growth.

Observed omega-3 fatty acid levels were increased in both MC4R mutants and ccβA-msEvol2/MC4R mutants when compared to controls, although the differences were not statistically significant. Observed EPA levels in MC4R mutants and ccβA-msEvol2/MC4R mutants were 94.12% and 92.16% higher than controls respectively. Observed levels of DHA levels in MC4R mutants and ccβA-msEvol2/MC4R mutants were 21.37% and 32.82% higher than

controls respectively. Overall observed levels of EPA+DHA were 50.00% and 5.72% higher in ccβA-msEvol2/MC4R fish than controls and MC4R fish. This indicates that both MC4R and elongase play a key role in omega-3 synthesis. As the MC4R mutation alone caused as high an increase in EPA as ccβA-msEvol2/MC4R together, it is possible that the increases in EPA were due to MC4R knockout rather than ccβA-msEvol2 insertion. Fish typically have higher levels of DHA than EPA, and elongase plays a more essential role in DHA synthesis than in EPA synthesis, potentially explaining the relatively larger increase in DHA in ccβA-msEvol2 fish.

To obtain the recommended 500mg of EPA+DHA per day, the consumer would need to eat approximately 275g of wild-type catfish. A typical serving of fish is 112g (Kris-Etherton et al., 2009). Thus, a consumer would need to eat nearly three servings to reach recommended levels. With the Evol2/MC4R genotype, a consumer would need to eat 1.64 servings of catfish per day to reach recommended levels of omega-3 fatty acids, half that of the wild type. While farmed Evol2/MC4R catfish still have EPA and DHA levels far below those of fatty marine fish such as salmon and tuna, they also have fewer of the issues associated with these fish including high mercury levels, microplastics and ecological disruption (Jackson et al., 2001; Burger et al., 2005; Lusher et al., 2017; Zupo et al., 2019).

The improved growth and omega-3 levels indicate that the use of ccβA-msEvol2/MC4R channel catfish could be beneficial for commercial farms. Catfish farming and production in the United States peaked in 2003, dramatically declined from 2007-2012 and has been gradually increasing since that time (Hanson and Sites, 2015; Torrans and Ott, 2018; FAO, 2020). Gene editing and transgenesis presents a valuable tool to increase profitability, sustainability and

industry growth. There are, however, a number of ethical, logistical and regulatory hurdles for the ccβA-msEvol2/MC4R mutant channel catfish to become applied commercially in the United States, as FDA currently regulates gene edited animals. The improvement of gene editing technologies, greater understanding of its effects and the commercial success of genetically improved organisms, including Aquabounty's AquAdvantage salmon make this technology a viable option in the near future. By combining transgenesis and gene editing with other genetic techniques, such as selection, crossbreeding and hybridization, it is likely possible to achieve even greater growth results, shorten the grow-out period as well as select for multiple traits. With an increasing human population and declining natural resources, all solutions should be evaluated to determine the most efficient and sustainable methods of food production.

References

Burger, J., Stern, A. H., & Gochfeld, M. (2005). Mercury in commercial fish: optimizing individual choices to reduce risk. *Environmental Health Perspectives*, *113*(3), 266-271.

Cheng, Q., Su, B., Qin, Z., Weng, C. C., Yin, F., Zhou, Y., ... & Dunham, R. A. (2014). Interaction of diet and the masou salmon Δ5-desaturase transgene on Δ6-desaturase and stearoyl-CoA desaturase gene expression and N-3 fatty acid level in common carp (*Cyprinus carpio*). *Transgenic Research*, *23*(5), 729-742.

Cuevas-Uribe, R., & Tiersch, T. R. (2011). Estimation of fish sperm concentration by use of spectrophotometry. Cryopreservation in Aquatic Species, 2nd edition. World Aquaculture Society, Baton Rouge, LA, 162-200.

Curtis, K. R., McCluskey, J. J., & Wahl, T. I. (2004). Consumer acceptance of genetically modified food products in the developing world. *AgBioForum*, 7(1&2): 70-75

Dunham, R.A. (2011). Aquaculture and fisheries biotechnology: genetic approaches. CABI Wallingford, Oxfordshire, UK

FAO (2020). The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome. https://doi.org/10.4060/ca9229en

Hanson, T. & Sites, D. (2015). 2014 U.S. Catfish Database. MSU AEC Information Report. Retrieved from http://www.agecon.msstate.edu/whatwedo/budgets/docs/catfish2014.pdf

Hasan, M. R., & Soto, S. Improving Feed Conversion Ratio and Its Impact on Reducing Greenhouse Gas Emissions in Aquaculture. FAO Non-Serial Publication 33 (FAO, Rome, 2017).

Huang, Y., W. Bugg, M. Bangs, G. Qin, D. Drescher, Nathan Backenstose, Chia Chen Weng, Yiliu Zhang, Karim Khalil, Sheng Dong, Ahmed Elaswad, Zhi Ye, Cuiyu Lu, Khoi Vo, R. M. Simora, X. Ma, Z. Taylor, Y. Yang, Tao Zhou, Jingping Guo, Guillaume Salze, Zhenkui Qin, Yi Wang, Rex A. Dunham (2021). Direct and Pleiotropic Effects of the Masou Salmon Delta5-Desaturase Transgene in F1 Channel Catfish (*Ictalurus punctatus*). *Transgenic Research*.

Jackson, J. B., Kirby, M. X., Berger, W. H., Bjorndal, K. A., Botsford, L. W., Bourque, B. J., ... & Warner, R. R. (2001). Historical overfishing and the recent collapse of coastal ecosystems. *Science*, *293*(5530), 629-637.

Jiang, D. N., Li, J. T., Tao, Y. X., Chen, H. P., Deng, S. P., Zhu, C. H., & Li, G. L. (2017). Effects of melanocortin-4 receptor agonists and antagonists on expression of genes related to reproduction in spotted scat, *Scatophagus argus*. *Journal of Comparative Physiology B*, *187*(4), 603-612.

Kris-Etherton, P. M., Innis, S., & Association, A. D. (2007). Position of the American Dietetic Association and Dietitians of Canada: dietary fatty acids. *Journal of the American Dietetic Association*, *107*(9), 1599-1611.

Lauritzen, L. (2001). The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. *Prog Lipid Res*, *40*, 1-94.

Liu, R., Kinoshita, M., Adolfi, M. C., & Schartl, M. (2019). Analysis of the role of the MC4R system in development, growth, and puberty of medaka. *Frontiers in Endocrinology*, *10*, 213.

Lusher, A., Hollman, P., & Mendoza-Hill, J. (2017). *Microplastics in fisheries and aquaculture: status of knowledge on their occurrence and implications for aquatic organisms and food safety*. FAO Fisheries and Aquaculture Technical Paper. No. 615. (FAO, Rome, 2017).

Meesapyodsuk, D., Reed, D. W., Covello, P. S., & Qiu, X. (2007). Primary structure, regioselectivity, and evolution of the membrane-bound fatty acid desaturases of *Claviceps purpurea*. *Journal of Biological Chemistry*, *282*(28), 20191-20199.

NMFS/NOAA (National Marine Fisheries Service – National Oceanic and Atmospheric Administration). (2013) Fisheries of the United States 2016. Current Fisheries Statistics No. 2016. NOAA, September 2013.

Owens L (2012) Diseases. In: Lucas JS, Southgate PC (eds) *Aquaculture: Farming Aquatic Animals and Plants*, pp. 214–228. John Wiley & Sons, New York, NY.

Peterman, M. A., & Posadas, B. C. (2019). Direct economic impact of fish diseases on the East Mississippi catfish industry. *North American Journal of Aquaculture*, *81*(3), 222-229.

Phelps, R. P., Hastey, R., Pendetar, A., Linley, L., Papanikos, N., & Dunham, R. A. (2007). Effects of temperature on the induced spawning of channel catfish and the production of channel× blue catfish hybrid fry. *Aquaculture*, *273*(1), 80-86.

Robinson, E. H., & Li, M. H. (2015). *Feed conversion ratio for pond-raised catfish*. Mississippi Agricultural & Forestry Experiment Station.

Saunders, A. V., Davis, B. C., & Garg, M. L. (2013). Omega-3 polyunsaturated fatty acids and vegetarian diets. *Medical Journal of Australia*, *199*, S22-S26.

Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedicine & Pharmacotherapy*, *56*(8), 365-379.

Smith, C. C., Harris, R. M., Lampert, K. P., Schartl, M., Hofmann, H. A., & Ryan, M. J. (2015). Copy number variation in the melanocortin 4 receptor gene and alternative reproductive tactics the swordtail *Xiphophorus multilineatus. Environmental Biology of Fishes*, *98*(1), 23-33.

Sprecher, H. (2000). Metabolism of highly unsaturated n-3 and n-6 fatty acids. *Biochem Biophys Acta*, *1486*, 219-231.

Tekedar, H. C., Karsi, A., Williams, M. L., Vamenta, S., Banes, M. M., Duke, M., ... & Lawrence, M. L. (2013). Complete genome sequence of channel catfish gastrointestinal septicemia isolate *Edwardsiella tarda* C07-087. *Genome Announcements*, *1*(6).

Tucker, C. (2012). Channel catfish. *Aquaculture Farming Aquatic Animals and Plants. 2nd Edition.* pp. 365-383. Wiley-Blackwell, Sussex, UK.

Wagner, B. A., Wise, D. J., Khoo, L. H. & Terhune, J. S. (2002) The Epidemiology of Bacterial Diseases in Food-Size Channel Catfish, *Journal of Aquatic Animal Health*, 14:4, 263-272

Wall, R., Ross, R. P., Fitzgerald, G. F., & Stanton, C. (2010). Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. *Nutrition Reviews*, *68*(5), 280-289.

Wang, S., Monroig, O., Tang, G., Zhang, L., You, C., Tocher, D. R., & Li, Y. (2014). Investigating long-chain polyunsaturated fatty acid biosynthesis in teleost fish: Functional characterization of fatty acyl desaturase (Fads2) and Elovl5 elongase in the catadromous species, Japanese eel *Anguilla japonica*. *Aquaculture*, *434*, 57-65.

Yoshimi, K., Kunihiro, Y., Kaneko, T., Nagahora, H., Voigt, B., & Mashimo, T. (2016). ssODN-mediated knock-in with CRISPR-Cas for large genomic regions in zygotes. *Nature Communications*, *7*(1), 1-10.

Zhang, X., Huang, J., Qiu, H., & Huang, Z. (2010). A consumer segmentation study with regards to genetically modified food in urban China. *Food Policy*, *35*(5), 456-462.

Zhou, T., Yuan, Z., Tan, S., Jin, Y., Yang, Y., Shi, H., ... & Gao, D. (2018). A review of molecular responses of catfish to bacterial diseases and abiotic stresses. *Frontiers in Physiology*, *9*, 1113.

Zupo, V., Graber, G., Kamel, S., Plichta, V., Granitzer, S., Gundacker, C., & Wittmann, K. J. (2019). Mercury accumulation in freshwater and marine fish from the wild and from aquaculture ponds. *Environmental Pollution*, *255*, 112975.

Chapter 5: Combining Ability for Body Weight of Fingerling and Food Size Channel Catfish, *Ictalurus punctatus,* Female X Blue Catfish, *I. furcatus*, Male Hybrid Progeny

Abstract

The objective of this study was to determine combining ability of channel catfish females, *Ictalurus punctatus,* and blue catfish, *I. furcatus*, males for body weight of their fingerling progeny grown in aquaria and food size fish progeny grown in earthen ponds using a 6 x 6 diallel design. The proportion of genetic variation compared to environmental variation decreased with age and size. At 14-months post hatch, the mean weight of the hybrid progeny was 9.69g±8.78g. The dam general combining ability ($GCA_{D,}$), sire general combining ability (GCA_S) and specific combining ability (SCA) were 4.5%, 8.4% 20.8% of total variance, respectively. The heaviest family was 700% and 38.95g larger than the lightest family ($p<2e-16$). At 40-months, at a mean weight of 836g±250.71, the $GCA_{D,}$ GCA_S and SCA for the interspecific matings were 1.1%, 0.0 and 4.6% of total variance, respectively. The observed mean of the heaviest family was 88% and 543.33g larger than the lightest family but was not significantly different ($p=0.414$). The heaviest and lightest families at 40-months post hatch were not the same as those at 14-months post hatch, indicating genotype-environment interactions. Environmental factors affected variation more than genetic factors for channel catfish female x blue catfish male hybrid at both life stages. When considering that the proportion of the genetic component of variation was overall the highest for SCA at both fingerling and food fish stages, reciprocal recurrent selection would be the best approach to further genetically improve hybrid body weight.

Keywords: Channel catfish, *Ictalurus punctatus,* blue catfish, *Ictalurus furcatus,* heritability, combining ability, growth, breeding, factorial design

1. Introduction

The basis for genetic improvement through breeding is means and variance (Bernardo, 2002). Assuming higher values of a trait are desired, a high mean for that trait in the starting population provides a head start and ensures that the desirable alleles are present. A large genetic variance allows for greater potential for genetic gain. Having a high mean and genetic variance are slightly contradictory however, as removing the bottom 50% of a population would increase the mean but decrease genetic variability. It is therefore essential to choose a population with a mean and variance that is appropriate for that particular genetic enhancement program. Choosing a high performing strain results in the best ending genetic product when conducting selection or utilizing other genetic enhancement program (Dunham and Smitherman 1983a, Dunham 2011). The Kansas strain is the oldest and one of the fastest growing domestic strains of channel catfish (Dunham and Smitherman 1983a, 1984), perhaps likely due to long domestication for growth, and some of the experimental fish were further growth enhanced through multiple generations of selection (Rezk et al. 2003, Dunham 2007, Dunham et al. 2008) . Utilizing the best performing strain or line is an important first step in a genetic enhancement program.

The genetic basis of interspecific hybridization is dominance, epistasis and overdominance. Combining ability is a measurement of how well strains, breeds, lines or species combine to produce heterosis (Griffing, 1956). General combining ability (GCA) informs the breeder which parents should be used in breeding programs by quantifying the additive genetic effects (Costa et al., 2018). Specific combining ability (SCA) can measure non-additive effects, such as dominance epistasis and heterosis and is useful in determining which genetic combinations perform better or worse than expectations based on the average performance of the parents. Some strains may show

sexual dimorphism for combining ability and therefore reciprocal crossbreeds often exhibit different performance. In the case of ictalurid catfish, this is likely due to genetic maternal effects. In channel catfish for example, Auburn strain females have good combining ability, while Auburn strain males do not (Dunham and Smitherman, 1983b). This maternal effect on combining ability is also apparent in interspecific hybridization of ictalurids, as 42 interspecific catfish hybrids have been assessed with the two best performing hybrids having channel catfish as the female parent.

In general, interspecific hybridization programs are unsuccessful for aquaculture application. Even if the many reproductive isolating mechanisms are overcome, heterosis is rarely achieved. The most commercially valuable exception is the channel catfish female x blue catfish male hybrid. Of the 42 North American catfish hybrids tested, only the channel catfish x blue catfish hybrid shows improvement in growth, disease resistance, low dissolved oxygen tolerance, dressing percentage and seinability (Smitherman and Dunham, 1985). However, while this hybrid is the best genotype for aquaculture, reproductive mechanisms hindered commercial utilization for nearly 4 decades. Channel catfish x blue catfish hybrids grow from fingerling to food sized fish 18-100% percent faster than either parent (Yant et al., 1975; Dunham and Smitherman, 1987; Dunham et al. 1990). However, the success of the hybrid varies by parental genotype and combining ability of the parents. Other examples of fish hybrids with improved growth include silver carp, *Hypophthalmichthys molitrix* x bighead carp, *Hypophthalmichthys nobilis* black crappie, *Pomoxis nigromaculatus* x white crappie, *Pomoxis annularis* (Hooe et al., 1994), and African catfish, *Clarias gariepinusi,* x Vundu, *Heterobranchus longifilis* (Salami et al., 1993; Nwadukwe, 1995).

Combining ability is an important predictor that can be used to determine if selection, crossbreeding or reciprocal recurrent selection are good options to produce genetic gain. Progeny from two separate factorial mating designs of channel catfish female x blue male catfish showed high dam general combining ability, intermediate sire general combining abilities and low dam x sire specific combining ability for both growth and carcass yield (Bosworth and Waldbieser, 2014). The data indicates that the genetic variance for harvest weight and carcass yield of hybrid catfish was mostly additive.

A diallel design involves a group of individuals to be used as male parents and a group of individuals to be used as female parents. In a diallel design, each male is mated with each female and each female is mated with each male so that all possible combinations of offspring are produced. While being resource intensive, especially with large numbers of crosses, it is useful in identifying strains, families or individuals with high breeding values, identifying crosses with good combining ability and potentially improving multiple traits at once. It is essential to have replicates of each cross in relevant simulated commercial environments to determine whether there is a genotype environment interaction and determine the best crosses for a particular environment, if the germplasm will be commercialized. Simulated models indicate that fully factorial models are more efficient at generating long term genetic variability and genetic response when compared to partial factorial, nested and single pair matings (Dupont-Nivet et al., 2006; D'Agaro et al., 2007). A higher number of offspring per mating pair is preferred (D'Agaro et al., 2007). The major drawback to factorial breeding programs is the high resource cost to maintaining a large number of genotypes.

The primary objective of this study was to determine combining ability for growth for fingerlings grown in aquaria and food fish cultured in earthen ponds for channel catfish female X blue catfish male hybrid catfish using a 6 x 6 diallel design, thereby allowing prediction of the bet potential genetic enhancement programs for growth improvement.

2. Materials and Methods

All experiments were conducted at the Fish Genetics Research Unit, E. W. Shell Fisheries Research Center, Auburn University, AL. All experimental protocols used in this experiment were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC) before the experiment was initiated and followed the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) protocols and guidelines.

2.1. Broodstock husbandry

Broodstock were cultured in 0.04-ha earthen ponds averaging 1-meter in depth. They were fed a 32 percent protein catfish pellet at 1-2% of their body weight five days per week. Dissolved oxygen was maintained above 3 mg/L using a ½ horsepower surface aerator (Air-O-Lator). Genotypes of fish are distinguished through various identification tools including Passive Integrated Transponder (PIT) tags, and heat brands. Individuals were chosen for spawning based on their health and secondary sexual characteristics.

2.2. Mating Design

For determination of interspecific combining ability, channel catfish females with Kansas ancestry were mated with Rio Grande strain blue catfish males. In 2017, three female Kansas Random strain and three female Kansas Mix strain (six females total) were each paired in a 6 X 6 diallel design with six male Rio Grande blue catfish for a total of 36 pairings.

2.3. Spawning

Male blue catfish, with large, muscular heads that were wider than the rest of their bodies and well-developed papilla were chosen. Dark coloring and scarring from territorial fighting were also signs of quality males in reproductive condition. Females with soft, well-rounded abdomen that were wider than their head, and a swollen urogenital opening were chosen. Broodstock were minimally handled and kept in tanks for as short a period of time as possible to limit stress.

Males were euthanized by a percussive blow to the head followed by pithing. The body cavity was opened carefully with a scalpel, ensuring not to pierce any organs. Testes were removed using tweezers and/or scissors and washed in a weigh boat with 0.9 percent saline using bottled distilled water, removing any blood or tissue. The water was drained, and the testes weighed before macerating the testes using scissors to release sperm. The homogenized testes were then filtered into a 50ml falcon tube using a 100-micron screen. The sperm was then diluted with 0.9 percent saline solution to a maximum of 10ml/gram of testes. Sperm concentration was tested using a Nanodrop 2000 Spectrophotometer (Thermo Scientific) and running a simple linear regression between sperm concentration and absorbance using the equation $y=-2.450+0.185\ln X$ (Adjusted $R2=0.601$) at 450nm (Cuevas-Uribe & Tiersch, 2011). Motility was analyzed under a 10X light microscope while sperm count was checked under 40X magnification.

Females were weighed and gently placed in labeled 32mm mesh bags. The spawning bags were then placed in a flow through holding tank so that the fish was fully submerged, ensuring that water quality was ideal with dissolved oxygen levels above 5 mg/L. A 14-gauge implanter was loaded with 100µg/kg body weight of luteinizing hormone releasing hormone analog (LHRHa)

implant. The needle was then inserted at a 45-degree angle ventrally adjacent to the pelvic fin and the implant was inserted. The ovulation time was predicted based on the degree-hours according to Phelps et al. (2007). Water temperature ranged from 26-28°C. Females were checked 36 hours after LHRHa injection and every four hours after that until ovulation. Once a female began ovulating, indicated by eggs visible on the bag, she was carefully transferred into a tank with 100 mg/L buffered tricaine methane sulfonate (MS-222) with equal parts sodium bicarbonate until completely anesthetized. Prior to this time, a thin layer of vegetable shortening was used to coat several 20cm diameter metal egg collection pans. The fish was then dipped in fresh water to wash off the MS-222 and gently dried with a towel. A thick layer of vegetable shortening, Crisco, was applied around the urogenital opening, including the pelvic fins to prevent sticking of the eggs during stripping. The female was hand stripped into the greased pan by gently applying pressure on the abdomen from the anterior end of the abdomen posteriorly towards the vent. Eggs would usually flow freely that were well-rounded and golden in color, with minimal blood. They were hand stripped ensuring no contact with freshwater, which can activate the eggs.

Approximately 3mL of sperm solution was added to the eggs and mixed gently. Fresh water was added to barely cover the eggs to activate the sperm and eggs, and the water was swirled to accomplish fertilization. After two minutes, the eggs should be fertilized, and after an additional 3 minutes, the spawning pan with the eggs was carefully submerged in a hatching trough with flow through water and a slow drip of calcium chloride. The eggs were left to harden for 1 hour. After 1 hour of hardening, the eggs were transferred into a labeled mesh basket suspended just below the surface in a 600L-hatching trough filled with flow through water and a slow drip of calcium chloride.

Paddlewheels were installed in the hatching troughs to circulate the water and gently agitate and aerate the eggs, mimicking the behavior of the male catfish parent. Water flow in the hatching troughs was maintained at ~25L/min, ensuring an exchange rate of 100%/25 min. Calcium chloride drips were placed at the upstream end of the hatching troughs to maintain hardness above 50ppm. Dissolved oxygen was maintained above 5ppm with compressed air and paddlewheels. Temperatures in hatching troughs were maintained between 24-28°C. Eggs were monitored daily for fungus growth. Treatment for fungus included manual removal of fungus and dead eggs and chemical treatment. Chemical treatments consisted of alternating 100ppm formalin and 32ppm copper sulfate static baths for 15 min every 8 hours. Chemical treatments were terminated 24 hours before expected hatch.

After about 5 days, or when the embryos are moving rapidly and close to hatch, fry catchers were placed around the mesh baskets to ensure newly hatched fry did not escape and mix within the tank. At the swim up stage, fry were transferred to 50L aquaria in recirculating systems until large enough to be PIT (Passive Integrated Transponders) tagged and moved out to ponds. Water quality was measured twice per week in the recirculating system.

2.4. Grow out and evaluation

Pellet feed size was adjusted to a maximum of ¼ the size of the mouth as the fish grew. Fry were fed Purina® AquaMax® powdered starter feed until they were large enough to eat Purina® AquaMax® 100. Both feeds contained 50% protein. All fish were fed daily to satiation. Fish were weighed at 14 months, PIT tagged and stocked communally in a 0.04-ha pond at

approximately 14,000 fish/ha. Fifteen fish per genetic type remained in the aquaria in the recirculating system. Fish were fed ad-libitum daily with 32% protein pelleted catfish feed. Fish were then harvested at 40-months post hatch, individually identified, weighed, sexed. During these growth trials any mortality due to disease was identified by family, genotype and sex, and the pathogen identified.

2.5. Statistical Analysis

Statistical analysis was conducted using SAS version 9.4 (Cary, NC, USA). Due to differential mortality in aquaria leading to differences in density, a regression between weight and density was calculated. The Proc Mixed function with a restricted estimation of likelihood was used to calculate the variance for the dam, sire, and dam × sire interactions and error.

General combining ability of the dams (GCA_D) equals the variance of the dams, calculated as the difference between the least squares means fitted for female parent effects in the model and the overall means of the progeny trial (Cotterill et al., 1986). Sire general combining ability (GCA_S) equals the variance of the sires. Specific combining ability, the proportion of variance due to the dam x sire interaction, was calculated as the means of the difference between the observed and predicted genetic values for each particular cross (Cotterill et al., 1986).

To compare the sources of variation in body weight, ANOVA and Tukey's multiple comparisons test was performed using R programming language (R Core Team, Vienna, Austria). Differences in variances between crosses were calculated using unpaired t-tests. Significance was tested at $\alpha = 0.05$.

3. Results

In 2017 a total of 1320 channel x blue hybrid catfish were generated from 36 families in a 6 x 6 diallel cross. At 14-months post hatch, the mean weight of the hybrid progeny was 9.69g±8.78g. The GCA_D estimate for body weight was 0.0021 and was responsible for 4.50% of total variance (Figure 24). The GCA_S estimate for body weight was 0.0040 and was responsible for 8.42% of total variance. The SGA estimate for body weight was 0.0098 and was responsible for 20.76% of total variance. The remaining 66.32% of variance was due to error. There were significant differences among mean body weights of families at 14-months post hatch ($p<2e-16$). At 14-months post hatch the heaviest family was 700% and 38.95g larger than the lightest family ($p<2e-16$).

At 40-months the mean weight was 836.64g±250.71. The GCA_D estimate for body weight was 0.0007 and was responsible for 1.09% of total variance (Figure 25). The GCA_S estimate for body weight was 0. The SGA estimate for body weight was 0.0031 and was responsible for 4.61% of total variance. The remaining 94.30% of variance was due to error. There were no significant differences between mean body weights of different families at 40-months post hatch ($p=0.243$). At 14-months post hatch the observed mean of the heaviest family was 88% and 543.33g larger than the lightest family, but was not significantly different ($p=0.414$). The heaviest and lightest families at 40-months post hatch were not the same as those at 14-months post hatch, indicating genotype-environment interactions.

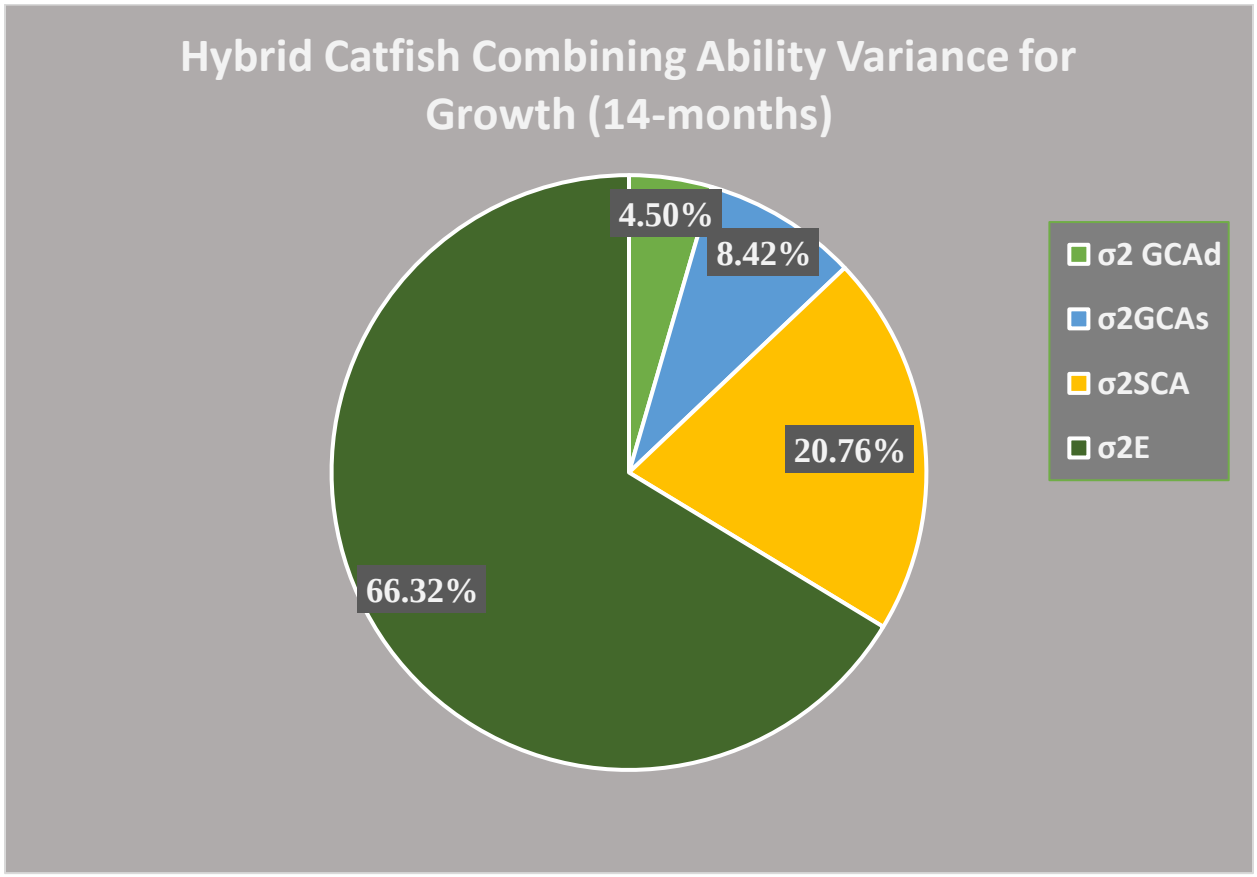

Figure 21. Comparison of the sire and dam general combining ability (GCA) and specific combining ability (SCA) estimate percentages for the female channel catfish (*Ictalurus punctatus*) X male blue catfish (*I. furcatus*) hybrid mating for growth at 14-months post hatch raised in 50L aquaria. The proportion of variation due to the dam, sire, dam x sire interaction and residual error is indicated by σ2GCAd, σ 2GCAds, σ2SCA, and σ2E, respectively.

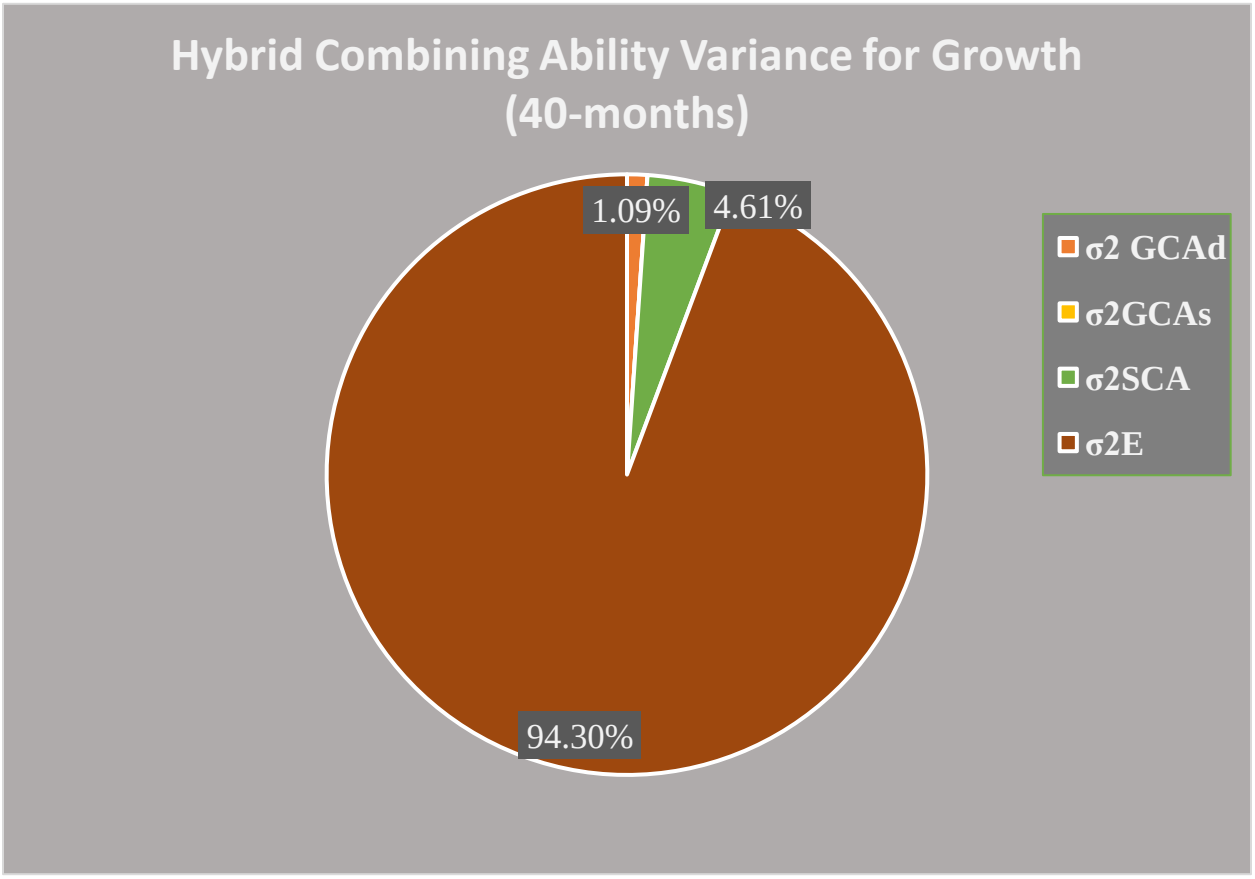

Figure 22. Comparison of the sire and dam general combining ability (GCA) and specific combining ability (SCA) estimate percentages for the female channel catfish (*Ictalurus punctatus*) X male blue catfish (*I. furcatus*) hybrid mating for growth at 40-months post hatch in 0.04ha earthen ponds stocked at 14,000 fish/ha. The proportion of variation due to the dam, sire, dam x sire interaction and residual error is indicated by σ2GCAd, σ 2GCAds, σ2SCA, and σ2E, respectively.

4. Discussion

In hybrid catfish, small fingerlings showed higher sire general combining ability, while market-sized fish displayed higher dam GCA. The variances in body weight explained by the dam, sire and dam x sire interaction at 14-months post were 4.50%, 8.42% and 20.78%, respectively. The remaining 66.32% of variance was explained by non-genetic factors. At 40-months post hatch the variances in body weight explained by the dam, sire and dam x sire interaction at 14-months post were 1.09 %, 0% and 4.61%, respectively, with the remaining 94.30% explained by non-genetic factors.

These results indicate a substantial genotype x environment interaction or genotype x age interaction. The relative importance of sire and dam effects changed with time and environment. Additionally, the heaviest and lightest families at 40-months post hatch were not the same as those at 14-months post hatch, indicating genotype-environment interactions. Environmental factors affected variation more than genetic factors for the channel catfish female x blue catfish male hybrid at both life stages, but environment played a much larger role at market size. When considering that the proportion of the genetic component of variation was overall the highest for SCA at both fingerling and food fish stages, reciprocal recurrent selection would be the best approach to further genetically improve hybrid body weight for Kansas x Rio Grande hybrids.

Odin (2017) also examined combining abilities in channel catfish female X blue catfish male hybrid catfish. Combining abilities were calculated for fish 311-days post hatch (dph) (mean weight 9.74g) grown in separate flow-through tanks, 556-dph (mean weight 91.25g) grown in separate recirculating aquaculture system (RAS) aquaria and 556-dph (mean weight

194.21g) grown communally in 0.05-ha earthen ponds. At 311dph, ratios of variances in body weight explained by dam, sire, dam x sire interaction and error were 31%, 9%, 10% and 50%, respectively. In fish grown in RAS aquaria to 556-dph variances in body weight explained by dam, sire, dam x sire interaction and error were 16%, 6%, 2% and 76%, respectively. In fish grown in earthen ponds to 556-dph variances in body weight explained by dam, sire, dam x sire interaction and error were 14%, 14%, 0% and 72%, respectively. The much higher variance due to the dam at small fingerling size in the study by (Odin, 2017) supports previous research indicating strong maternal effects during early development of teleosts (Heath et al., 1999; Berkeley et al., 2004; Green, 2005; McCormick, 2005). In both the previous study (Odin, 2017) and the current study, maternal effects decreased substantially over time, although there was a greater reduction in relative percentages in the current study. Variance in body weight due to paternal effects, however, increased in Odin (2017), while they decreased to 0% in the current study. While SCA decreased over time in both studies, SCA fell to 0% when grown in communal earthen ponds (Odin, 2017), while SCA was the largest genetic contributor of variance in the current study. In both studies, non-genetic factors contributed a larger role on variances in body weight at later stages, although the shift was far more dramatic in the current study. While Odin (2017) did not report combining abilities past the sub-market stage, it is possible that genetic factors would play an increasingly smaller role in body weight variance as the fish continued to grow.

Rapid growth during early development in fish is essential for predator avoidance and survival and the genetic contributions by parents to growth, particularly the dam, are strongest at early stages (Metcalfe and Monaghan, 2003). As a fish develops, environmental factors

increasingly influence growth, and therefore, body weight in the population body weight should become more homogeneous.

Combining abilities can vary widely depending on species and genotype. Costa et al. (2018) found that Serrasalmidae fingerlings from 8 genetic groups, including 3 species and 5 hybrids, had a cumulative body weight GCA and SCA ratio of 56.80% and 3.95%, respectively, with the remaining 39.25% due to non-genetic factors. However, GCA was not broken down into dam and sire components. At market size, the GCA and SCA ratios were 52.37% and 13.13% with the remaining 34.50% due to non-genetic factors. These results indicate that additive effects can have large effects on hybrid body weight in some genera of hybrids.

Bosworth and Waldbieser (2014) also found that genetic variance for body weight was primarily additive in hybrid catfish. At a mean weight of 639.4g, variances in body weight explained by dam, sire and dam x sire interaction were 25%, 8% and 2%, respectively with 65%, of variation due to non-genetic effects. Bosworth and Waldbieser (2014) used D&B strain blue catfish males and unknown strains of channel catfish females from three commercial farms. Combining ability estimates vary depending on the genotype of parents and cannot be generalized within a species. In this case, different strains of channel catfish and blue catfish were used compared to the current study, fry were transferred to ponds at a young age and stocking densities may have varied.

Genetic diversity and number of parents in the study may be important as well. In the current study, only one lineage of channel catfish, one strain of blue catfish and the least number

of parents were utilized, resulting in the smallest combining abilities observed among all similar studies. Channel catfish from three farms and a different strain of blue catfish (Bosworth and Waldbieser, 2014) and multiple strains of both channel catfish and blue catfish (Odin, 2017), along with more parents in both cases compared to the current study, were utilized, resulting in larger combining abilities.

In a highly competitive market, reciprocal recurrent selection for fast growing hybrid catfish fingerlings could provide a farmer with an advantage. Combining abilities for channel catfish female X blue catfish male hybrid catfish matings appear to vary due to genetic type, age and culture environment. Thus, combining abilities would need to be generated for each specific case to identify the best hybrid catfish genetic enhancement program at each hatchery. Future experiments should evaluate combining abilities for more strains of ictalurid catfish to determine optimal combinations of channel catfish females and blue catfish males for a variety of culture environments.

References

Berkeley, S. A., Chapman, C., & Sogard, S. M. (2004). Maternal age as a determinant of larval growth and survival in a marine fish, *Sebastes melanops*. *Ecology*, *85*(5), 1258-1264.

Bernardo, R. (2002). *Breeding for quantitative traits in plants*. Vol. 1, p. 369. Stemma press. Woodbury, MN

Bosworth, B., & Waldbieser, G. (2014). General and specific combining ability of male blue catfish (*Ictalurus* furcatus) and female channel catfish (*Ictalurus punctatus*) for growth and carcass yield of their F1 hybrid progeny. *Aquaculture*, *420*, S29-S35.

Cotterill, P. P., Dean, C. A., & Van Wyk, G. (1987). Additive and dominance genetic effects in *Pinus pinaster, P. radiata* and *P. elliottii* and some implications for breeding strategy. *Silvae Genetica*, *36*(5-6), 221-232.

Costa, A. C., Botelho, H. A., Gomes, R. C. D. S., de Sousa Campos, S. A., Reis Neto, R. V., Balestre, M., ... & de Freitas, R. T. F. (2019). General and specific combining ability in Serrasalmidae. *Aquaculture Research*, 50(3), 717-724.

Cuevas-Uribe, R., & Tiersch, T. R. (2011). Estimation of fish sperm concentration by use of spectrophotometry. Cryopreservation in Aquatic Species, 2nd edition. World Aquaculture Society, Baton Rouge, LA, 162-200.

D'agaro, E., Woolliams, J. A., Haley, C. S., & Lanari, D. (2007). Optimizing mating schemes in fish breeding. *Italian Journal of Animal Science*, *6*(sup1), 795-796.

Dunham, R. A. (1995). The contribution of genetically improved aquatic organisms to global food security. In *International Conference on the Sustainable Contribution of Fisheries to Food Security, Kyoto (Japan), 4-9 Dec 1995*.

Dunham, R. A., & Smitherman, R. O. (1983a). Crossbreeding channel catfish for improvement of body weight in earthen ponds. *Growth*, *47*(1), 97-103.

Dunham, R. A., & Smitherman, R. O. (1983b). Response to selection and realized heritability for body weight in three strains of channel catfish, *Ictalurus punctatus*, grown in earthen ponds. *Aquaculture*, *33*(1-4), 89-96.

Dunham, R. A., Smitherman, R. O., & Goodman, R. K. (1987). Comparison of mass selection, crossbreeding, and hybridization for improving growth of channel catfish. *The Progressive Fish-Culturist*, *49*(4), 293-296.

Dunham, R. A. 2007. Comparison of six generations of selection, interspecific hybridization, intraspecific crossbreeding and gene transfer for growth improvement in ictalurid catfish. *Aquaculture*. 272 (supplement 1): S252-S253.

Dunham, R. A., G. M. Umali, R. Beam, A. H. Kristanto and M. Trask. 2008. Comparison of production traits of the NWAC103 channel catfish, NWAC103 channel X blue hybrid catfish, Kansas select 21 channel catfish and blue catfish grown at commercial densities and exposed to natural bacterial epizootics. *North American Journal of Aquaculture*. 70:98-106.

Dupont-Nivet, M., Vandeputte, M., Haffray, P., & Chevassus, B. (2006). Effect of different mating designs on inbreeding, genetic variance and response to selection when applying individual selection in fish breeding programs. *Aquaculture*, *252*(2-4), 161-170.

Gjedrem, T. (2000). Genetic improvement of cold-water fish species. *Aquaculture Research*, *31*(1), 25-33.

Green, B. S., & McCormick, M. I. (2005). Maternal and paternal effects determine size, growth and performance in larvae of a tropical reef fish. *Marine Ecology Progress Series*, *289*, 263-272.

Griffing, B. (1956a). Concept of general and specific combining ability in relation to diallel crossing systems. *Australian Journal of Biological Science*, 4, 463–493.

Heath, D. D., Fox, C. W., & Heath, J. W. (1999). Maternal effects on offspring size: variation through early development of chinook salmon. *Evolution*, *53*(5), 1605-1611.

Kincaid, H. L., Bridges, W. R., & Von Limbach, B. (1977). Three generations of selection for growth rate in fall-spawning rainbow trout. *Transactions of the American Fisheries Society*, *106*(6), 621-628.

Metcalfe, N. B., & Monaghan, P. (2003). Growth versus lifespan: perspectives from evolutionary ecology. *Experimental gerontology*, *38*(9), 935-940.

Odin, R. Y. (2017). Combining Ability of Channel Catfish, Ictalurus punctatus, Females and Blue Catfish, I. furcatus, Males for Early Growth, Growth Variation, Disease Resistance, and Carcass Yield of their Progeny. Doctoral dissertation, Auburn University, AL, USA.

Phelps, R. P., Hastey, R., Pendetar, A., Linley, L., Papanikos, N., & Dunham, R. A. (2007). Effects of temperature on the induced spawning of channel catfish and the production of channel× blue catfish hybrid fry. *Aquaculture*, *273*(1), 80-86.

Smitherman, R. O., & Dunham, R. A. (1985). Genetics and breeding. *Channel Catfish Culture. Elsevier Scientific Publishing, Amsterdam*, 283-316.

Yant, D. R., Smitherman, R. O., & Green, O. L. (1975). Production of hybrid [blue *Ictalurus furcatus* (Lesueur) male, channel *I. Punctatus* (Rafinesque) female] catfish and channel catfish in earthen ponds. M. S. Thesis, Auburn University, AL.