Comparisons of Growth Hormone Transgenic Channel Catfish (*Ictalurus punctatus*), and Channel Catfish Transgenic for Gonadotropin Releasing Hormone Knockout Genes Grown in Earthen Ponds at High Density

by

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

Auburn, Alabama
December 15, 2018

Key Words: growth hormone gene, antisense gene, transgenic sterilization, sex maturity score, growth rate

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Abstract

The objective of the experiment was to compare the growth rate and sexual maturity score of growth hormone transgenic channel catfish, transgenic sterilization channel catfish, and non-transgenic channel catfish at high density, similar to a modular catfish culture system. Fish were harvested at 4 years of age and at 5 years of age after being communally grown in the same pond. Sex-age interactions were observed. CAB (tilapia GnRH antisense transgene) females had the highest observed early body weight but were the smallest genetic type of female at final harvest. PAB (rainbow trout GnRH antisense transgenic) had the highest observed body weight at final harvest for females. GAD (goldfish glutamate decarboxylase transgene) had the highest early body weight for males. AFP (channel catfish growth hormone gene transgenic) had the highest observed final body weight and the highest observed body weight gain between years 4 and 5. Sexual maturity was highly and positively correlated with growth rate. GnRH antisense transgenic channel catfish had reduced growth rate and sexual development. Alternative genetic sterilization techniques or the current technology coupled with selection may be needed to overcome this negative pleiotropic effect.

Acknowledgments

I want to express my deepest gratitude to my advisor Dr. Rex Dunham for accepting me into the Genetics Lab and for his guidance, patience and support all of the time. I want to thank my committee members Dr. Jeffery Terhune and Dr. Charles Chen for their time and guidance the last two years. I want to thank of all my lab mates for their friendly help and encouragement. Special thanks should go to Chia Chen Weng Chen for her continuous correction and assistance in the lab. I owe my fish health to David Creamer because of his support and correction in greenhouse and pond work. My special appreciation goes to Dr. Nermeen Youssef Abass Youssef for her insightful comments, helpful guidance and encouraging words.

I also want to thank my home school, Shanghai Ocean University, for their joint program with Auburn University and financial support. I appreciate the research guidance from teachers of Hunan Agriculture University from when I was a college student, which sparked my enthusiasm for fisheries. In addition, I am grateful to Dr. Cova Arias for giving me the opportunity to come to Auburn.

I want to express my gratitude to my parents for their unconditional love and support for twenty-five years and further on. Sincere thanks for their sparing no effort in giving me the best chance to receive an education. Thanks go to my Auburn Family for their support and even the prayers for me.

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List of Abbreviations

FAO Food and Agriculture Organization of United States

USDA United States of Department of Agriculture

AFP antifreeze protein

GH growth hormone

ccGH channel catfish growth hormone

GAD glutamate decarboxylase

GnRH Gonadotropin-releasing hormone

LH Luteinizing hormone

LSH Follicle-stimulating hormone

GH Growth Hormone

Introduction

The world population had reached 7.7 billion by October of 2018. It will keep growing up to 11.2 billion until 2100 (UN, 2018). Food and fiber yield cannot meet the demands of the rapidly increased population with non-GMO plants and animals (Ouchi & McCluskey, 2003). Genetically modified organisms (GMO) have been a novel food and fiber resource in numerous countries throughout the world during the last 3 decades. Aquaculture is the most rapidly growing industry in the world. Fish produced by aquaculture accounts for at least 50% of the world's fish consumption (FAO, 2018). Aquaculture is now known as one of the most promising industries in the world as fish provide a variety of superior and healthy animal protein. Fishing is still main fish resource, accounting for 47% of world fish production and aquaculture production accounting 53% of world fish production (FAO, 2018). However, current catchment every year has been over twice the value estimated to guarantee sustainable fishing (Coll, Libralato, Tudela, Palomera, & Pranovi, 2008). Overfishing of ocean can lead to species extinction and other problems, including pollution, degradation of water quality, and anthropogenic climate change (Jackson et al., 2001). Therefore, improvements of aquaculture production are essential with the reduction of natural fisheries resources due overfishing and environmental degradation.

Catfish has the leading role in aquaculture in North America. According to the nutrition facts report, catfish is a good resource of protein, containing 51% of protein at dried catfish fillet. Catfish is also rich in vitamin B, niacin, pantothenic acid and phosphorus as well as small amounts of potassium, copper, magnesium, iron and zinc (USDA, 2016). Most catfish farms in the US are located in the southeastern states, including Alabama, Arkansas, Mississippi, and Texas. Water surface has been reduced by at least 71% by 2018 (NASS,

2018) after reaching its peak of 79.6 thousand acres in 2002 (NASS, 2002). Therefore, improvements of aquaculture production are essential with the reduction of resources.

In the United States, channel catfish, *Ictalurus punctatus*, was originally the most farmed catfish because of its superior growing ability, ease in spawning, high tolerance of handling, disease resistance and tolerance of low dissolved oxygen and poor water quality (Kelly, 2004).

Transgenic technology is a tool to improve culture traits of catfish. Scientists started working on transferring genes by molecular genetic technology after the breakthrough of gene recombination (Lederberg & Tatum, 1946). The next breakthrough in genetic enhancement in the commercial aquaculture industry may be through transgenesis. Much of the early research on transgenic fish focused on growth hormone gene transfer (Devlin et al., 2001; Du et al., 1992; Martine et al., 1996).

Growth hormone is a polypeptide synthesized by anterior pituitary to accelerate growth of the body and maintain nitrogen, mineral, lipid and carbohydrate metabolism (Walter, 1983). Transgenic salmon containing all-fish chimeric growth hormone driven by an antifreeze promoter grew 3-6 X larger than controls with the largest transgenic fish being 13 X that of the control (Du et al., 1992). Growth-enhanced transgenic Atlantic salmon, *Salmo salar*, grew 2.62 to 2.85 times larger the non-transgenic full siblings (Cook, McNiven, Richardson, & Sutterlin, 2000). Growth enhancement of 7-month-old transgenic tilapia with chinook salmon growth hormone gene driven by AFP promoter was three more times compared to their non-transgenic full-sibling (Rahman, Mak, AYAD, Smith, & Maclean, 1998). GH transgenic rohu (*Labeo Rohita*) driven CMV promoter showed more than 4 times growth enhancement than their non-transgenic siblings while GH transgenic rohu with beta-actin promoter showed 4.5X and 5.8X growth enhancement (Venugopal, Anathy, Kirankumar, & Pandian, 2004). Transgenic mud loaches (*Misgurnus mizolepis*) grew 35

times faster and larger than controls (Nam et al., 2001). In common carp, *Cyprinus carpio*, transgenic fish were 1.96 times larger than the largest full sibling control by 4 months of age (Wang, 2000). Transgenic Yellow River carps (*Cyprinus carpio*) containing an "all fish" construct CAgcGH, grass carp growth hormone gene driven by common carp β-actin promoter also showed enhancement in growth rate and food conversion efficiency (Wu, Sun & Zhu, 2002). The utilization of the proper promoter is key to successful genetic engineering. Antifreeze protein promoter (AFP) has been used in several successful GH transgenic fish experiments (Du et al., 1992; Cook et al., 2000; Hobbs & Fletcher, 2008; Hew & Fletcher, 1996). Ocean pout (*Zoarcidae americanus*) can produce several kinds of antifreeze proteins that can protect fish from freezing when the surrounding temperature declines below the freezing point of their body fluids (Hew et al., 1988). These antifreeze proteins consisted of different combinations and arrangements of different amino acids that also show annual changes with seasonal temperature changes (Fletcher, Hew, Li, Haya & Kao, 1985). Scientists transferred bacterial CAT gene driven by AFP promoter, which demonstrated that promoter regions of AFP gene can function after transferring to a new organism (Gong, Hew & Vielkind, 1991). AFP-ccGH transgenic fish showed great tolerance to sub-zero temperatures and osmoregulation ability (Abass et al., 2016). At -0.5℃, AFP-ccGH transgenic catfish had 100% survival rate while AFP-ccGH controls, channel catfish, GAD transgenic fish, GAD controls and hybrid catfish only had 0-2% survival rate at 0 ppt salinity. With the salinity raised to 2.5 ppt at the same temperature, AFP-ccGH transgenic channel catfish still showed better survival rate than other genetic groups (Abass et al., 2016).

The potential environmental risk of these fast growing transgenic fish impedes the approval and application of these fish in commercial aquaculture. Although transgenic fish demonstrated lower fitness (Hedrick, 2001; Dunham, 2004; Dunham et al., 1994), reproduction and sexual maturity (Dunham et al., 1992; Chatakondi, 1995), poorer predator

avoidance (Dunham, 1995), swimming ability (Farrell, 1997), and foraging ability (Chitmanat, 1996), there is still concern that transgenic fish may cause some harm to wild fish populations including ecosystem displacements, disruption or species extinctions. If these escaped fish are sterile, some potential negative effects caused by transgenic fish would be manageable (Devlin, Sundström, & Muir, 2006). Thus, there is a need for sterile transgenic fish program development. Potential genetic means includes hybridization, polyploidy and transgenic sterilization. Surgical sterilization can be used in small quantity of important fish because of high labor and time cost. Hybridization, monosex populations and triploids all have the weakness of requiring fertile brood stock that could escape and cause permanent environmental impact. Repressible transgenic sterilization is a better choice as well as offspring of escaped brood stock would be sterile preventing long-term impact (Li et al., 2017). Transgenic sterilization projects may have some effects on fish growth rate because of interaction between GnRH and GH.

Gonadotropin-releasing hormone (GnRH) is one of the most important hormones released by hypothalamus. The main function of GnRH is to stimulate the pituitary to synthesis and release the follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Campbell, Gaidamaka, Han, & Herbison, 2009). FSH and LH are related to gonad development by regulating germ cell proliferation and differentiation. Therefore, most transgenic sterilization researches worked on inhibiting or reducing the synthesis and secretion of GnRH (Zohar, Gothilf & Wray, 2007; Ye, 2017; Qin et al., 2016). GnRH is also a neurohormone regulated by many neurons with different transmitters including GABA and glutamate (Trudeau et al., 2000). Transgenically, disrupting the production of gonadotropin releasing hormone (GnRH) followed by hormone therapy could be one mechanism to sterilize catfish, and then restore fertility on demand (Ye, 2017). Glutamic acid decarboxylase (GAD) can simulate the decarboxylation of glutamate to γ-aminobutyric acid

(GABA) and CO_2. In channel catfish, reproduction endocrine regulation can be disrupted by overexpressing the GAD gene (Ye, 2017), as theoretically, the overexpression of GAD prevents the proper migration of GnRH neurons in the developing embryo (Vastagh et al., 2014).

Another potential mechanism of transgenically preventing the expression of GnRH gene is antisense technology. Antisense technology is a powerful technology to regulate gene expression. Antisense oligonucleotides block translation of target mRNAs in a sequence specific manner, either by steric blocking of translation or by destruction of the bound mRNA via RNase-H enzyme (Sahu, Shilakari, Nayak & Kohli, 2007). There have been many achievements using antisense for functional genomics (Hamilton, Lycett & Grierson 1990; Ayub et al., 1996) and antisense gene therapy (Zhang, Jeong, Boado, & Pardridge, 2002a; Zhang, Y., Zhu, C., & Pardridge, 2002b). Gonadally deficient and completely sterile transgenic common carp carrying antisense sGnRH RNA driven by a carp β-actin promoter were generated, suggesting an effective method to produce transgenic fish (Hu et al., 2007). Uzbekova et al. found that transgenic rainbow trout carrying antisense GnRH RNA driven by Pab promoter were not infertile even through the production of the endogenous GnRH mRNA in the brain and pituitary was reduced (2000). Physiologically reversible fertile transgenic common carp carrying GnRH cDNA and antisense GnRH cDNA driven by common carp β-action promoter was generated in 2004 (Li, 2004).

GnRH and GH can interact and affect the expression of each other. GnRH peptides could increase the serum GH level for forty-eight hours after a single intraperitoneal injection with increased body weight after several times of injection (Marchant, Chang, Nahorniak & Peter, 1989). sGnRH could stimulate goldfish, *Carassius auratus*, pituitary to synthesis and release GH in vitro (Marchant et al., 1989). Sex steroids could stimulate the release of GH through a perifusion system on common carp. Serum GH of the sexually mature fish and

sexually-regressed fish showed more powerful response to sGnRH than those immature fish (Lin, H. et al., 1995). sGnRH and sGnRH-A could function as GH-releasing factors while somatostatin could function as GH-releasing inhibitor (Lin, X., Lin, H., & Peter, 1993). Reciprocally, GH could also regulate GnRH pituitary and the gonadotropin response via negative feedback effect of testosterone in adult male transgenic mice bearing human GH gene (Chandrashekar, Bartke, Wagner, 1988). Anderson et al., (2006) pointed out "There was a feedback loop was found in fish (i) LH induction of GH release from somatotrophs, (ii) amplification of GH secretion by GH autoregulation in somatotrophs, and (iii) GH feedback inhibition of LH release from neighboring gonadotrophs." Yao et al. found growth hormone receptors in ovary, testis, fat, skin, cartilage, gill, blood pellet, brain, spleen, kidney, and muscles in rainbow trout (1991). Le Gac et al found GH binding and action directly in trout testis. These binding sites were similar to GH receptors found in liver. Salmon GH and bovine GH could even modulate steroidogenesis in vitro (Le Gac, Ollitrault, Loir, & Le Bail, 1992). This experiment was also conducted on *fundulus heteroclitus* by Singh et al. (1988). In immature rainbow trout, GH level can be raised by boosting estradiol via decrease SRIF-14 (Holloway et al. 1997c). In sexual mature rainbow trout, increased endogenous GH levels were observed (Sumpter, Lincoln, Bye, Carragher, & Le Bail, 1991; Foucher, Le Bail, & Le Gac, 1992). Holloway and Leatherland found GH levels and sexual maturation, timing of ovulation and steroid levels are correlated in rainbow trout (1997d). In vitro, the gonadal steroid priming of immature rainbow trout increases pituitary responsiveness to GH secreagogues (GnRH) (Holloway and Leatherland, 1997a). Similar results have been found on mature rainbow trout (Holloway and Leatherland, 1997b).

AFP-ccGH channel catfish was designed to get growth enhancement by overexpressing the channel catfish gene, which was generated by inserting channel catfish GH driven by AFP promoter into channel catfish genome. GnRH was reduced by excess GABA generated

by overexpressed GAD65 gene in GAD project, in which GAD65 gene driven by carp β-actin promoter was inserted into channel catfish genome. In PAB and CAB project, GnRH antisense technology was used to inhibit the function of GnRH gene. In PAB project, Atlantic salmon GnRH antisense gene driven by corresponding Pab promoter was inserted into channel catfish genome to prevent GnRH gene functioning. In CAB project, tilapia GnRH antisense gene driven by carp β-actin promoter was inserted into channel catfish to prevent GnRH gene from functioning.

AFP, CAB, PAB, GAD, and non-transgenic channel catfish controls were cohabitated at in the same pond at high density for 5 years. The primary objective was to compare the growth performance and sexual maturity development of F1 generation of GAD, CAB, PAB and non-transgenic channel catfish.

2 Materials and Methods

2.1 Transgene constructs

AFP-ccGH construct was comprised of growth hormone cDNA of channel catfish (accession number: NM_001200245) and the promoter (accession number: AY594644.1) and termination (AY594644.1) regions derived from an ocean pout. The size of growth hormone cDNA size was 603 bp. The size of antifreeze protein promoter 5' was 2,120 bp. The size of 3' terminator was 1,161 bp. (Figure 1) (Abass, 2016).

βA-GAD65 construct was goldfish glutamate decarboxylase 65 (Accession number: AF045594) driven by carp β-actin promoter. The GAD65 gene was 2,653 bp and the carp ß-actin promoter was 6,782 bp (Figure 1) (Abass, 2016).

The pPCab-IfS plasmid consisted of the tilapia GnRH antisense gene and common carp β-actin promoter. This total plasmid was 8,546 bp (Figure 2).

The Pab-sGnRH plasmid construct consisted of antisense DNA complementary to Atlantic salmon GnRH cDNA driven by the Pab promoter derived from the corresponding

GnRH gene. The total length was 3,066 bp with the 2,487bp of promoter and 577 bp of GnRH antisense gene (Figure 3) (Uzbekova, 2000).

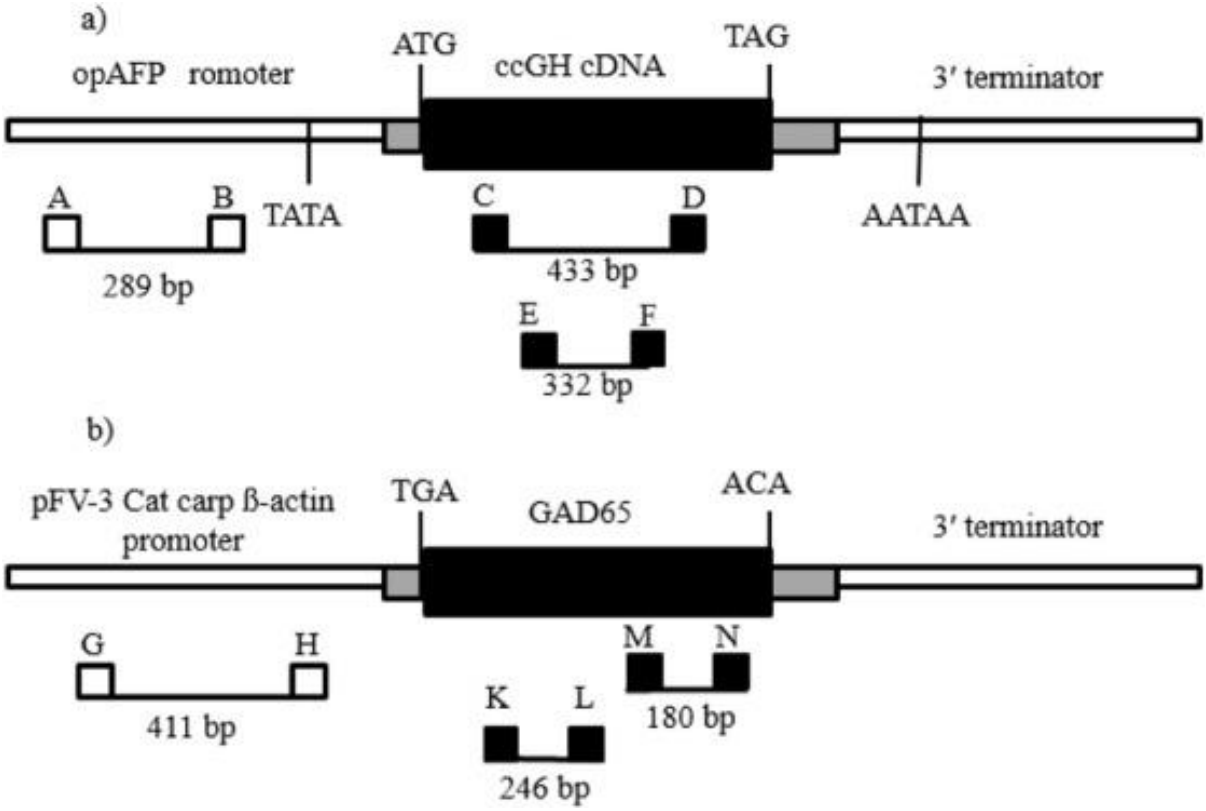

Figure 1 Schematic representation of AFP-ccGH and pFV-3 Cat carp β-actin promoter construct (Abass, 2017). (a) ocean pout, *Zoarces americanus*, AFP gene promoter and 3' terminator region; ▬ channel catfish, *Ictalurus punctatus*, (growth hormone) GH cDNA, ▭ 5' and 3' untranslated regions. (b) Structure of the goldfish glutamate decarboxylase 65 (GAD65): ▭ pFV-3 CAT Common carp, *Cyprinus capio*, β-actin promoter and 3' terminator region; ▬ goldfish, *Carassius auratus*, glutamate decarboxylase 65 gene, ▭ 5' and 3' untranslated regions.

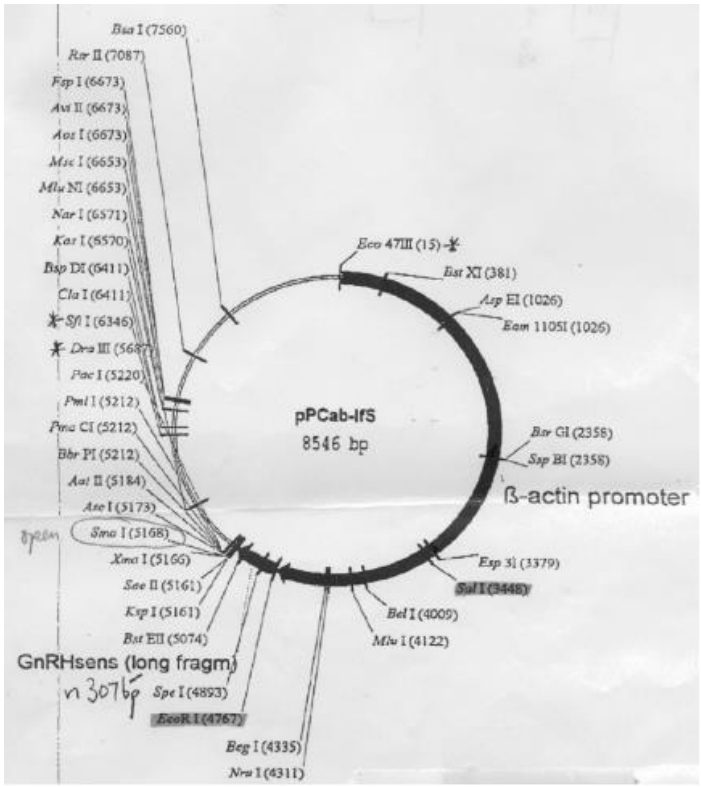

Figure 2 Schematic representation of pPCab-IfS construct. Carp, *Cyprinus carpio*, β-actin promoter and tilapia GnRH antisense gene (long fragment).

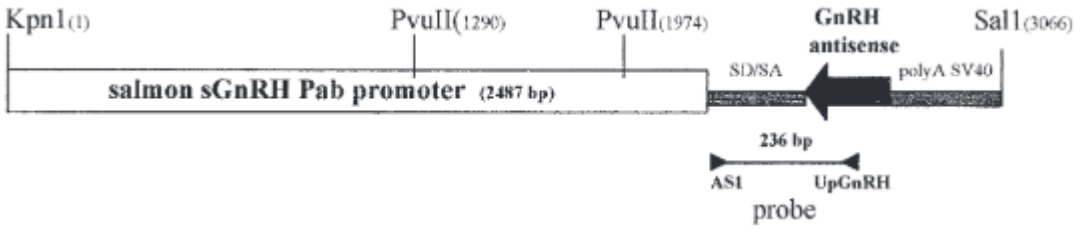

Figure 3 Schematic representation of Pab-sGnRH antisense construct. Atlantic salmon, *Salmo salar*, sGnRH Pab promoter, Atlantic salmon GnRH antisense gene and polyadenylation signal (Uzbekova et al., 2000)

2.2 Broodstock spawning

The brood fish were from the Fish Genetics Research Unit, School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University, AL, USA. The brood fish were harvested from ponds and separated in holding tanks by sex with continuous water flow. All the fish weights, pit tags and brands were recorded. The females were kept in separate mesh bags. These females received 2 injections of luteinizing hormone releasing hormone (LHRHa). The first dose was injected at a rate of 0.1mL/kg of the concentration of 200 μg/mL followed by the second dose at the same rate at the concentration of 1,000 μg/mL after 12 hours. Eggs were released 36-48 hours after the second dose.

Testes were harvested, cleaned with saline and macerated manually. Sperm were placed in tubes with saline as the diluent after filtration to remove tissue. The tubes had holes in the lids to allow the sperm to breathe and were stored at 4℃.

2.3 Fertilization

Eggs were stripped into pans. Sperm solution was applied to eggs at the rate of 7mL/50g of eggs. A small amount of pond water was added to each pan at the rate of 20mL/50g of eggs. Additional pond water was added to the fertilized eggs 3-5 minutes after fertilization.

2.4 Incubation and hatching

Eggs were incubated in mesh baskets in paddlewheel hatching troughs with continuous water flow. Formalin solution was applied to the hatching trough prevent fungus every 8 hours statically for 30 minutes at 70 ppm during the first 3 days of incubation to prevent fungus. Heaters were used to maintain the water temperature at approximately 27 degrees Celsius. On the 4^{th} day, small mesh baskets were utilized to prevent fry escaping upon hatching. Dead eggs were removed twice a day. Hardness, nitrate, nitrite, alkalinity, chloride, ammonia, dissolved oxygen (D.O.) and pH were monitored, and were maintained at 75 ppm, 0 ppm, 0 ppm, 0 ppm, 0ppm, 0 ppm, saturation level and 7.2.

2.5 Fish culture

F1 generation was produced in June of 2013. Fries were stocked in the early morning when the water temperature was under 28 degree Celsius and the mean weight of each group was approximately 0.16g. Fish were stocked communally in a single pond at the density of 109,316 fish/ha (Table 1) and fed ad-libitum every day. Algae blooms were monitored while feeding. D.O. was monitored and corrected by aeration every day. It should be over 3.0 ppm. Ammonia, Nitrite, Nitrate, Alkalinity, pH and hardness were measured and recorded weekly or when problems were suspected. In 2017, 753 fish were harvested and stocked into pond G70 at the density of 18,800 fish/ ha and cultured as before.

2.6 Data collection

Fish were harvested in April of 2017 at 4 years of age and April of 2018 at 5 years of age. Weight, sex, pit tag and sexual maturity score were recorded. Sexual maturity was graded by analyzing many different characteristics. Females were judged by gravidness (softness and protruding of abdomens), the presence of red, swollen, mucoid, genital papillae. Males were judged by width of head, presence of muscle on the top of the head, presence of fighting scars, darkness of color, leanness and largeness and distinction of genital papillae. The degree of female sexual maturation score was categorized as: Score 1: no difference between females and males from external appearance; Score 2: not gravid enough to spawn, apparent linear genital opening; Score 3: smaller abdomen, fullness can be felt on palpation, vent with a smaller genital opening generally white in color; Score 4: large abdomen, firm on palpation, small vent but pinkish in coloration; and Score 5: fully distended, very large round abdomen, soft on palpation, pinkish vent, and protruding large genital opening (Chatakondi, Yant, Kristanto, Umali-Maceina & Dunham, 2011). The degree of male sexual maturation score was categorized as: Score 1: no difference between females and males from external appearance; Score 2: closed genital papillae, lean body shape; Score 3: lean body, dark color,

apparent but white genital papillae; Score 4: Dark color, lean body, widen head, apparent muscle on top of head, distinct genital papillae; and Score 5: Dark color, lean body, widen head, strong muscle on top of head, and distinct and big genital papillae.

Pelvic fins were sampled for DNA analysis. DNA were extracted by proteinase K with protein precipitation and ethanol (Appendix 1). The concentrations and qualities of DNA were analyzed with a NanoDrop. All DNA were diluted into 500ng/mL before PCR. Mutations were detected by polymerase chain reaction (PCR). DNA samples were amplified with the primers (in Table 2 & Table 3).

PCR products were tested by 1% gel. The length of PCR products was shown through Imagelab.

2.7 Statistical analysis

Data analysis of weight and sexual maturity score were analyzed with RStudio software. Data were presented as mean, coefficient of variation and percentage difference from the controls to evaluate the influences of genotype. One-way ANOVA was used to test the effect of each factor. Student's t test was used to compare the difference between transgenic fish and non-transgenic controls.

Table 1 Mean body weight of transgenic PAB, AFP, CAB, GAD, and non-transgenic control channel catfish, *Ictalurus punctatus*, grown in ponds at 109,316 fish/ha at 2 weeks old. Families were not genotyped prior to stocking.

[1]Gene	[2]N	Mean weight/g
AFP	500	0.164
AFP	30	0.166
AFP	90	0.166
AFP	350	0.160
GAD	1,000	0.150
GAD	1,000	0.100
GAD	200	0.150
PAB	150	0.190
PAB	500	0.100
CAB	300	0.170

[1]PAB - channel catfish transgenic for Atlantic salmon, *Salmo salar*, GnRH antisense gene driven by salmon sGnRH Pab promoter. AFP - channel catfish transgenic for channel catfish growth hormone gene driven by ocean pout antifreeze protein promoter. CAB - channel catfish transgenic for tilapia GnRH antisense gene driven by common carp, *Cyprinus carpio*, β-actin promoter. GAD - channel catfish transgenic for goldfish, *Carassius auratus*, glutamate decarboxylase driven by common carp, β-actin promoter.

[2]N = Number of population size;

Table 2 Primer sequences used for the amplification of AFP gene promoter and channel catfish (*Ictalurus punctatus*) growth hormone in channel catfish. Amplified sizes were also shown.

Primer	Primer direction	Sequence (5'-3')	Amplified size	Target regions
AFP1	F	CCTCAGATAAGCGGAGGAAA	290bp	AFP promoter
AFP2	F	TGACCCGACCTCAGATAAGC	282bp	AFP promoter
AFP6	R	GGATGCGCTTAAGACCTTTG	290bp(1F6R) 282bp(2F6R)	AFP promoter
GH1	F	GCCAAGATGATGGACGACTT	414bp	Channel catfish GH cDNA
GH1	R	GAGACCTATCTGAGCGTGGC	414bp	Channel catfish GH cDNA
GH2	F	AGGAAGCTCTGTTGCCTGAA	313bp	Channel catfish GH cDNA
GH2	R	CTACCAGACCTTGAGCGAGG	313bp	Channel catfish GH cDNA

AFP-ccGH-channel catfish transgenic for catfish growth hormone gene driven by the ocean pout, *Zoarces americanus*, antifreeze protein gene promoter

F = Forward primer, R = Reverse primer.

Table 3 Primer sequences used for the amplification of GAD, CAB, and PAB in transgenic channel catfish (*Ictalurus punctatus*). Amplified sizes were also shown.

Primer name and direction	Sequence 5'-3'	Amplified size	Description
1F	TTGCTFFCACATCTGAG	274bp	[2]GAD
1R	TACAATCACACCTGTCCAA	274bp	GAD
1F	GCGGCCGCTAATGGTTGCCT	247bp	[3]CAB
3F	CTTTCCACCTGGTAGCCATC	208bp	CAB
3R	AGTGTCTGGTGATGCTGTGC	247bp(1F3R)	CAB
		208bp(3F3R)	
3F	TGGTGGTGCAAATCAAAGAA	231bp(3F4R)	[4]PAB
		245bp(3F6R)	
4R	TCCCATGGATCTTAGCAACA	231bp	PAB
6R	GCCGCCGGAGAAACTCCCAT	245bp	PAB

[1]F = Forward primer, R = Reverse primer;

[2]GAD - channel catfish transgenic for goldfish, *Carassius auratus*, glutamate decarboxylase driven by common carp, *Cyprinus carpio*, β-actin promoter;

[3]CAB - channel catfish transgenic for tilapia GnRH antisense gene driven by common carp β-actin promoter;

[4]PAB - channel catfish, *Ictalurus punctatus*, transgenic for Atlantic salmon, *Salmo salar*, GnRH antisense gene driven by salmon sGnRH Pab promoter.

3 Results

At 4 years of age, AFP males had observed mean body weight of 10.58% higher than controls, which was not significant (p=0.72) and a single female was 8.39% larger (Table 4). At 5 years of age, the AFP males were 19.86% larger than male controls (p= 0.32), but the AFP female died or lost her pit tag (Table 5). GAD males were 34.21 % larger than male controls (p=0.10), but GAD females were 11.50% (p=0.10) smaller than controls at 4 years of age (Table 4), which were not significant different. However, transgenic male growth slowed for GAD as they aged and the difference between GAD and wild type was -6.77% (p=0.65) and -7.40% (p=0.51) for males and females, respectively, at 5 years of age and was not different from controls. PAB males were also larger, 33.19% (p=0.22), than control males at 4 years of age, while females were 4.55% (p=0.97) larger than their controls (Table 4). Again, these relationships changed as the PAB males were 9.12% (p=0.34) smaller than non-transgenic males at 5 years of age, while females were 9.84% (p= 0.35) larger (Table 5). In the case of CAB, transgenic males were 3.67% (p= 0.86) smaller than wild type males at 4 years of age (Table 4), while CAB females were 23.06% (p= 0.18) larger than their controls. Age effects were apparent again as CAB males were 28.47% (p=0.27) smaller and CAB females 12.50% (p=0.27) smaller than non-transgenic channel catfish when 5 years of age (Table 5).

Table 4 Mean body weight, standard error of the mean, coefficient of variation, percent difference compared to the control, range of body weight and p-value of Student's t-test of transgenic PAB, AFP, CAB, GAD, and non-transgenic control channel catfish, *Ictalurus punctatus,* grown in ponds at 18,800 fish/ha at 4 years of age. Values of the weights represent the Mean±2SEM.

Body Weight(g)							
[1]Genetic type	Sex	[2]N	Mean± 2[3]SEM	[4]CV	[5]% difference from control	Range	[6]P
	Mixed	34	190.68±50.08	76.04	+19.05	Max 790.0 Min 70.0	0.23
PAB	Female	15	163.97±34.72	41.00	+4.55	Max 280.0 Min 70.0	0.97
	Male	19	216.82±84.54	84.80	+33.19	Max 790.0 Min 70.0	0.22
	Mixed	11	179.09±82.74	76.62	+11.81	Max 510.0 Min 70.0	0.65
AFP	Female	1	170.00±0.00	0.00	+8.39	-	-
	Male	10	180.00±91.25	80.39	+10.58	Max 510.0 Min 70.0	0.72
	Mixed	17	171.71±39.94	47.71	+7.20	Max 380.0 Min 50.0	0.60
CAB	Female	7	193.00±46.60	31.88	+23.06	Max 268.0 Min 72.0	0.18
	Male	10	156.80±65.16	65.72	-3.67	Max 380.0 Min 50.0	0.86

	Mixed	51	175.49±49.14	76.89	+9.56	Max 720.0 Mix 40.0	0.43
GAD	Female	25	138.80±55.52	52.20	-11.50	Max 280.0 Min 40.0	0.10
	Male	26	218.46±85.68	75.84	+34.21	Max 720.0 Min 60.0	0.10
	Mixed	470	160.17±14.78	58.01	-	Max 771.0 Min 10.0	-
Non-transgenic controls	Female	206	156.84±21.86	50.88	-	Max 770.0 Min 40.0	-
	Male	264	162.78±20.04	62.70	-	Max 771.0 Min 10.0	-

[1]PAB - channel catfish transgenic for Atlantic salmon, *Salmo salar,* GnRH antisense gene driven by salmon sGnRH Pab promoter. AFP - channel catfish transgenic for channel catfish growth hormone gene driven by ocean pout, *Zoarces americanus*, antifreeze protein promoter. CAB - channel catfish transgenic for tilapia GnRH antisense gene driven by common carp, *Cyprinus carpio*, β-actin promoter. GAD - channel catfish transgenic for goldfish, *Carassius auratus*, glutamate decarboxylase driven by common carp β-actin promoter. [2]N = Number of sample size;

[3]SEM = Standard error of the mean;

[4]CV = Coefficient of variation;

[5]% difference from controls = (Mean of transgenic – Mean of non-transgenic) × 100% / Mean of non-transgenic;

[6]P = from Student's t-test with the non-transgenic control;

[7]* = Significant codes from F test of all groups. *** = 0.001. ** = 0.01. * = 0.05.

Table 5 Mean body weight, standard error of the mean, coefficient of variation, percent difference compared to the control, range of body weight, survival and p-value of Student's t-test of transgenic PAB, AFP, CAB, GAD, and non-transgenic control channel catfish, *Ictalurus punctatus*, grown in ponds at 18,800 fish/ha at 5 years old. Values of the weights represent the Mean±2SEM.

			Body Weight (g)					
Genetic Groups	Sex	N	Mean ±2[2]SEM	[4]CV	% difference from control	Range	P	% Survival year 4-5
PAB	Mixed	23	453.69 ±82.55	43.63	+11.75	Max 884.0 Min 29.0	0.65	67.65
	Female	15	454.80 ±79.29	33.76	+9.84	Max 643.0 Min188.0	0.35	100.00
	Male	8	451.63 ±194.92	61.04	-9.12	Max 884.0 Min 29.0	0.34	42.11
AFP	Mixed	9	595.61 ±232.17	58.47	+46.70	Max 1,127.0 Min 182.0	0.27	81.82
	Female	0	-	-	-	-	-	0.00
	Male	9	595.61 ±232.17	58.47	+19.86	Max 1,127.0 Min 182.0	0.32	90.00
CAB	Mixed *	16	355.27 ±89.55	50.41	-12.50	Max 634.0 Min 154.0	0.03	94.12
	Female *	7	355.07 ±96.44	35.91	-14.25	Max 522.0 Min 225.0	0.27	100.00
	Male *	9	355.44 ±149.37	63.03	-28.47	Max 634.0 Min 154.0	0.08	90.00

	Mixed	31	478.35 ±105.49	61.39	+17.82	Max 1,162.0 Min 31.0	0.70	60.78
GAD	Female *	11	383.45 ±89.96	38.90	-7.40	Max 584.0 Min 170.0	0.51	44.00
	Male	20	530.55 ±152.55	64.30	+6.77	Max 1,162.0 Min 31.0	0.65	76.92
	Mixed	355	406.00 ±25.39	58.91	-	Max 2,798.0 Min 156.0	-	75.53
Non-transgenic controls	Female *	163	414.06 ±23.39	36.07	-	Max 817.0 Min 160.5	-	79.13
	Male	192	496.93 ±41.94	58.48	-	Max 2,798.0 Min 156.0	-	72.73

[1]PAB - channel catfish transgenic for Atlantic salmon, *Salmo salar*, GnRH antisense gene driven by salmon sGnRH Pab promoter. AFP - channel catfish transgenic for channel catfish growth hormone gene driven by ocean pout, *Zoarces americanus*, antifreeze protein promoter. CAB - channel catfish transgenic for tilapia GnRH antisense gene driven by common carp, *Cyprinus carpio*, β-actin promoter. GAD - channel catfish transgenic for goldfish, *Carassius auratus*, glutamate decarboxylase driven by common carp β-actin promoter;

[2]N = Number of sample size;

[3]SEM = Standard error of the mean;

[4]CV = Coefficient of variation;

[5]% difference from controls = (Mean of transgenic – Mean of non-transgenic) × 100% / Mean of non-transgenic;

[6]P = from Student's t-test with the non-transgenic control;

[7]* = Significant codes from F test of all groups.

*** = 0.001. ** = 0.01. * = 0.05.

Table 6 Mean body weight gain of transgenic PAB, AFP, CAB, GAD, and non-transgenic control channel catfish, *Ictalurus punctatus*, grown in ponds at 18800 fish/ha from 4-5 years old. Values of the weights represent the Mean±2SEM.

Body Weight gain (g)							
Genetic Groups	Sex	N	Mean±2SEM	[4]CV	% difference from control	Range	P
PAB	Mixed	22	315.14±54.52	40.57	-0.68	Max 587.0 Min 48.0	0.94
	Female	15	297.27±60.90	39.67	10.69	Max 470.0 Min 48.0	0.37
	Male	7	353.43±112.78	42.21	-0.72	Max 587.0 Min 187.0	0.97
AFP	Mixed	10	413.38±145.71	55.73	30.28	Max 837.0 Min 132.0	0.25
	Female	-	-	-	-	-	-
	Male	10	413.38±145.71	55.73	16.12	Max 837.0 Min 132.0	0.25
CAB	Mixed **	16	195.06±71.08	72.88	-38.53	Max 474.0 Min 15.0	0.00
	Female **	7	162.07±76.17	62.18	-39.65	Max 292.0 Min 15.0	0.03
	Male	9	220.72±112.72	76.60	-38.00	Max 872.0 Min 68.0	0.04

	Mixed	28	359.32±82.99	61.11	13.24	Max 872.0 Min 68.0	0.33
GAD	Female	11	239.82±52.02	35.97	-10.70	Max 384.0 Min 120.0	0.31
	Male	17	436.65±119.35	56.35	22.65	Max 872.0 Min 68.0	0.20
	Mixed	461	317.31±14.72	49.79	-	Max 1,090.0 Min 15.0	-
Non-transgenic controls	Female*	204	268.57±14.43	38.38	-	Max 582.0 Min 15.0	-
	Male	257	356.00±22.67	51.05	-	Max 1090.0 Min 24.0	-

[1]PAB - channel catfish transgenic for Atlantic salmon, *Salmo salar*, GnRH antisense gene driven by salmon sGnRH Pab promoter. AFP - channel catfish transgenic for channel catfish growth hormone gene driven by ocean pout, *Zoarces americanus*, antifreeze protein promoter. CAB - channel catfish transgenic for tilapia GnRH antisense gene driven by common carp, *Cyprinus carpi*, β-actin promoter. GAD - channel catfish transgenic for goldfish, *Carassius auratus*, glutamate decarboxylase driven by common carp β-actin promoter.

[2]N = Number of sample size;

[3]SEM = Standard error of the mean;

[4]CV = Coefficient of variation;

[5]% difference from controls = (Mean of transgenic – Mean of non-transgenic) × 100% / Mean of non-transgenic;

[6]P = from t test with the non-transgenic control;

[7]* = Significant codes from F test of all groups. *** = 0.001. ** = 0.01. * = 0.05.

The body weight gain of CAB transgenic males and females were 39.65% and 38.00% less than non-transgenic males and females ($p<0.05$). The body weight gain of PAB transgenic males and females were 10.69% ($p=0.37$) and -0.72% ($p=0.97$) higher than non-transgenic males and females. The observed body weight gain of AFP transgenic males was 30.28% higher than non-transgenic males. The body weight gain of GAD transgenic males and females were -10.70% ($p=0.31$) and 22.65% ($p=0.20$) higher than non-transgenic males and females, which were not significant different (Table 6).

At five years of age, AFP males showed sexual maturity grade that was 49.38% higher than controls ($p=0.07$). The sexual maturity grade of PAB females was 18.75% higher than the non-transgenic females ($p=0.08$). The sexual maturity grade of PAB males was 21.25% higher than the non-transgenic males but not significantly different ($p=0.44$). Sexual maturity of mixed sex PAB transgenic project was 19.38% higher than the non-transgenic controls, ($p=0.08$). In the case of CAB, the mean sexual maturity grade of CAB transgenic males was 29.38% lower than the control males ($p<0.05$). The mean sexual maturity grade of CAB females was 6.25% lower than the control males but not significantly different ($p=0.75$). The mean sexual maturity grade of CAB project fish was 44.62% higher than controls ($p<0.05$). The mean sexual maturity of GAD transgenic females and males were 5.00% ($p=0.71$) and 9.38% ($p=0.48$) higher than the non-transgenic control females and males, respectively, which were not significantly different from controls. The sexual maturity of mixed sex GAD was 8.13% higher than the non-transgenic group, and not significantly different ($p=0.42$) (Table 7).

Table 7 Mean sex maturity score, standard error of the mean, coefficient of variation, percent difference compared to the control, p-value of Student's t-test with controls of transgenic PAB, AFP, CAB, GAD, and non-transgenic control channel catfish, *Ictalurus punctatus*, grown in ponds at 18,800 fish/ha at 5 years of age. Values of the weights represent the Mean±2SEM.

Sex maturity score							
[1]Genetic types	Sex	[2]N	Mean± 2[3]SEM	[4]CV	[5]% difference from controls	Range	[6]P
PAB	Mixed	23	1.91±0.34	42.86	+19.38	Max 4.0 Min 1.0	0.08
	Female	15	1.90±0.31	31.58	+18.75	Max 2.5 Min 1.0	0.08
	Male	8	1.94±0.83	60.82	+21.25	Max 4.0 Min 1.0	0.44
AFP	Mixed	9	2.39±0.76	47.70	+49.38	Max 5.0 Min 1.5	0.07
	Female	-	-	-	-	-	-
	Male	9	2.39±0.76	47.70	+49.38	Max 5.0 Min 1.5	0.07
CAB	Mixed [7]***	16	1.30±0.29	44.62	-18.75	Max 2.5 Min 0.5	0.04
	Female *	7	1.50±0.57	50.00	-6.25	Max 2.5 Min 0.5	0.75
	Male ***	9	1.13±0.22	29.20	-29.38	Max 1.5 Min 0.5	0.00

	Mixed ***	31	1.73±0.31	49.13	+8.13	Max 3.5 Min 0.5	0.42
GAD	Female *	11	1.68±0.43	42.86	+5.00	Max 3.0 Min 0.5	0.71
	Male *	20	1.75±0.42	53.71	+9.38	Max 3.5 Min 0.5	0.48
	Mixed **	35 5	1.60±0.07	43.75	-	Max 4.0 Min 0.25	-
Non-transgenic Controls	Female **	16 3	1.60±0.10	38.75	-	Max 3.5 Min 0.5	-
	Male **	19 2	1.60±0.11	47.5	-	Max 4.0 Min 0.25	-

[1]PAB - channel catfish transgenic for Atlantic salmon, *Salmo salar*, GnRH antisense gene driven by salmon sGnRH Pab promoter. AFP - channel catfish transgenic for channel catfish growth hormone gene driven by ocean pout, *Zoarces americanus*, antifreeze protein promoter. CAB - channel catfish transgenic for tilapia GnRH antisense gene driven by common carp, *Cyprinus carpio*, β-actin promoter. GAD - channel catfish transgenic for goldfish, *Carassius auratus*, glutamate decarboxylase driven by common carp β-actin promoter.

[2]N = Number of sample size;

[3]SEM = Standard error of the mean;

[4]CV = Coefficient of variation;

[5]% difference from controls = (Mean of transgenic – Mean of non-transgenic) × 100% / Mean of non-transgenic;

[6]P = from t test with the non-transgenic control;

[7]* = Significant codes from F test of all groups. *** = 0.001. ** = 0.01. * = 0.05.

Body weight and sexual maturity score were related (Table 8). The body weight and sexual maturity score of PAB males and females had strong correlation ($r=0.906$, $p<0.05$) and moderately strong correlation ($r=0.703$, $p<0.05$). The body weight and sexual maturity development of the whole PAB project had strong correlation ($r=0.832$, $p<0.05$). The body weight and sexual maturity development of AFP males had strong correlation ($r=0.844$, $p<0.05$). The body weight and sexual maturity development of CAB males and females had weak correlation ($r=0.495$, $p=0.176$) and moderately strong correlation ($r=0.735$, $p=0.060$). The body weight and sexual maturity development of CAB had weak correlation ($r=0.477$, $p=0.162$). The body weight and sexual maturity development of GAD females and males had weak correlation ($r=0.424$, $p=0.194$) and moderately strong correlation ($r=0.644$, $p<0.05$). The body weight and sexual maturity development of GAD had weak correlation ($r=0.590$, $p<0.05$). The correlation between the body weight and sexual maturity score of non-transgenic females and males was very weak ($r=0.353$, $p<0.05$) and weak ($r=0.593$, $p<0.05$). The correlation between the body weight and sexual maturity score of the non-transgenic group was moderately strong ($r=0.709$, $p<0.05$).

Table 8 Correlation coefficient between body weight and sexual maturity transgenic PAB, AFP, CAB, GAD, and non-transgenic control channel catfish, *Ictalurus punctatus*, grown in ponds at 18,800 fish/ha at 5 years old.

[1]Genetic Groups	Sex	Correlation Coefficient	[2]p-value
PAB	Mixed	0.832	0.000
	Female	0.703	0.003
	Male	0.906	0.002
AFP	Mixed	0.844	0.004
	Female	-	-
	Male	0.844	0.004
CAB	Mixed	0.477	0.062
	Female	0.735	0.060
	Male	0.495	0.176
GAD	Mixed	0.590	0.000
	Female	0.424	0.194
	Male	0.644	0.002
Non-transgenic controls	Mixed	0.709	0.000
	Female	0.353	0.000
	Male	0.593	0.000

[1]PAB - channel catfish transgenic for Atlantic salmon, *Salmo salar*, GnRH antisense gene driven by salmon sGnRH Pab promoter. AFP - channel catfish transgenic for channel catfish growth hormone gene driven by ocean pout, *Zoarces americanus*, antifreeze protein promoter. CAB - channel catfish transgenic for tilapia GnRH antisense gene driven by common carp, *Cyprinus carpio*, β-actin promoter. GAD - channel catfish transgenic for goldfish,

Carassius auratus, glutamate decarboxylase driven by common carp β-actin promoter;

[2]p-value of correlation coefficient.

In the 4th year, AFP group, PAB group, GAD males, CAB females showed growth enhancement while CAB males and GAD females didn't show growth enhancement, which were not significantly different. In the 5th year, PAB females, AFP males, GAD males showed growth enhancement while CAB fish, PAB males and GAD females didn't show growth enhancement, which were not significant different ($p>0.05$). The sex maturity score of PAB, AFP and GAD project fish were higher than the non-transgenic controls, which were not significantly different. CAB project fish showed lower growth development than the non-transgenic controls while only CAB males showed lower sexual development significantly. At the same time, the correlation coefficient also showed that there will some relationship between the weight and sexual maturity score. The correlation coefficient of CAB project fish and GAD females showed that there was fairly positive relationship between weight and sexual developmental which were not significantly different. In AFP populations, PAB population and GAD male populations, the weight and sexual maturity development were positively related ($p<0.05$) (Table 8).

4 Discussion

Sex-age interactions and rate of sexual maturity had major impacts on the observed growth of transgenic channel catfish. CAB females were the largest at sub-market size after 4 years of growth at high density, which is analogous to the modular system of growing channel catfish (D'Abramo et al., 2008). However, their growth slowed as they were the smallest genetic type of female after an additional year of growth. CAB males were the smallest male genetic type at the conclusion of both growth phases. PAB females were moderate in body weight after the first growth phase, but their growth accelerated and were

the largest females at 5 years of age, whereas, PAB, males had the opposite growth pattern, changing from one of the largest genetic types at 4 years of age to one of smallest at 5 years of age. GAD males were relatively fast growing during the entire growth experiment, but GAD females were one of the slower growing genetic types in both growth phases. AFP males had moderate growth rates in the initial phase but were the largest genetic type by the conclusion of the second growth phase. Non-transgenic controls exhibited moderate growth throughout the experiment. The growth enhancement observed were less than that observed previously for GH transgenic channel catfish (Abass, 2016; Abass et al., 2016).

This might be due to genotype-environment interactions associated with high density culture. It is more difficult for all fish to access feed in high density culture environments and associated severe water quality problems, leading to under feeding, genotype-environment interactions potentially caused by transgene silencing. Genetic information is encoded not only by the linear sequence but also by epigenetic modification of chromatin structure. The most common epigenetic mechanism are DNA methylation and histone modification. Environmental stressors can affect the epigenetic patterns and effect changes in gene activation, resulting gene silencing (Meister & Tuschl, 2004). Transgenic fish had lower competence in foraging ability at high density in a cohabitated environment with non-transgenic fish (Devlin, D'Andrade, Uh, & Biaji, 2004). Transgenic fish were 3.7 times as long as the wild and restricted hormone gene transgenic fish as well as 26 times as heavy as the wild and the restricted growth transgenic fish. The restricted transgenic fish and the wild fish had the similar growth rate (Sundström, Lõhmus, Tymchuk & Devlin, 2008).

A poor aquaculture environment may also cause DNA methylation or histone modification. In many animals, including humans, early life stress can have a long lasting influence on animals later life and even their offspring (Heim & Binder, 2012). Confirming statistical differences among genetic types was difficult even though % differences were

relatively large. This was likely due to the high level of variation induced by the high density. In some cases, there were individuals within genetic types with apparent stunted growth rates. The CV for body weight was much higher than what is usually observed. There were PAB individuals that were 10-15% larger than the largest control, and GAD and AFP individuals that were 50% larger than the largest control. This could be indicative of considerable family effects, which would contribute to overall variation. The next generation of these transgenic catfish have the potential for much greater growth under high density conditions if these largest individuals were successfully selected and their phenotypic performance transmitted to the next generation.

Low replication for the transgenic types also contributed to the confounding of the statistical analysis. This could have been caused by differential mortality in the initial culture of these fish or low inheritance rates.

The sexual development and body weight of channel catfish were positively correlated both within and among genetic types, and was particularly strong among genetic types, r=0.86-0.88. This may present a significant problem to overcome for practical application when genetic sterilization is desired. The fish with the most sexual development had the best growth. If these fish show sexual maturity traits without fertility, perhaps these transgenic sterilization programs will not negatively impact growth. However, the results from the current study suggest that presumed suppression of GnRH reduces growth rate, potentially counteracting some of the benefits of growth enhancement. Li et al. (2018) also found suppression of gamete production, in fact the complete knockdown of gonad development, which may produce important steroid hormones needed for growth, via knockdown of primordial germ cells, reduced growth and survival by 25%. Li et al. (2018) found a subset of sterile individuals with outstanding growth, thus, again selection may enable the solving of this problem.

Additional experiments should be conducted to confirm the growth results and growth patterns. The transgenesis coupled with selection should be evaluated to determine its potential for maximizing and standardizing the phenotypic enhancement. The role of GH and GnRH levels should be examined carefully to more fully explain the results observed in this experiment, and how that might affect future strategies to simultaneously increase growth while controlling reproduction. Epigenetic effects such as those induced by DNA methylation should be explored across generations to assist in explaining the variable results and to develop mechanisms to reduce variation.

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Appendix 1

1 The protocol to make Holthfreter's solution

1.1 Holthfreter's stock solution

1,050g of NaCl, 60g of $NaHCO_3$ and 15g of KCl were added to 10L of city water.

1.2 $MgSO_4$ stock solution.

150g of $MgSO_4$ were added to 500mL of city water.

1.3 Doxycycline stock solution

1.5g of Doxycycline were added to 30mL of water.

1.4 Holthterfreter's working solution protocol

2L Holthterfreter's stock solution, 40mL $MaSO_4$ stock solution, 20mL KCl Stock solution, 12ml Doxycycline stock solution were added to water, making the 60L solution.

1.5 Artemia incubation protocol

3g artemia eggs were added to 3ppt of water for 24-36 hours.

2 DNA extraction

1 10-20mg pieces from fins were cut.

2 Cell lysis buffer (600μL/sample) and 20mg/mL of proteinase K solution (3μL/sample) were applied to each sample.

3 Each sample and solution were mixed by vortexing for 10 sec.

4 Samples were incubated in the water base for 3 hours.

5 170 μL of protein precipitation Solution were applied to each sample. Each sample were Samples were vortexed vigorously for 20 sec then they were incubated on ice for 15 min.

6 Each sample was centrifuged at 15000rpm/min for 10 min at room temperature.

7 Supernatant was poured into new tubes. 600 μL of Isopropanol were added to each tube. Solutions in each tube were mixed by inverting over 15X.

8 Each sample was centrifuged again at 15000 rpm/min for 5 min at room temperature.

9 600 μL 70% ethonal were used in each tube to wash the DNA pallet. This procedure was repeated.

10 The DNA pallets were dried in the air for 3 hours. 50 μL water was added to melt the DNA.

Appendix 2

Table 9 Water surface used for catfish production (thousand acres)-States and Uniteds:1989-2018

1989		1990		1991		1992		1993		1994		1995		1996		1997		1998	
Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul
139	140	150	158	162	166	162	158	152	149	152	154	155	159	167	162	177	164	173	165
1999		2000		2001		2002		2003		2004		2005		2006		2007		2008	
Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul
175	172	189	179	190	186	197	185	187	175	178	170	174	158	170	156	163	151	155	132
2009		2010		2011		2012		2013		2014		2015		2016		2017		2018	
Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul	Feb	Jul
147	112	115	95	100	95	90	78	83	*	76	64	70	57	62	55	61	54	62	56