Gene Editing and Hormone Therapy to Control Reproduction in Channel Catfish, *Ictalurus punctatus*

by

Guyu Qin

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Approved by

Rex Alan Dunham, Chair, Professor of School of Fisheries, Aquaculture, and Aquatic Sciences
Joanna Wysocka-Diller, Associate Professor of Biological Sciences
Terrill Hanson, Professor/Extension Specialist of Fisheries, Aquaculture, and Aquatic Sciences
Timothy Braden, Associate Professor of Anatomy, Physiology and Pharmacology

Abstract

Channel catfish (*Ictalurus punctatus*) and their hybrids account for more than 60% of aquaculture production in the US. The catfish industry is being severely challenged during recent years as a result of higher operating costs, disease, and competition from cheaper imported frozen catfish. A potential future tool to help address this problem is the utilization of genetically engineered catfish. To sterilize channel catfish, transcription activator-like effector nuclease (TALEN) was applied to induce mutagenesis of catfish type gonadotropin-releasing hormone (cfGnRH) and follicle-stimulating hormone (FSH) genes through electroporation respectively. Zinc finger nuclease (ZFN) was performed to knock out the luteinizing hormone (LH) gene through electroporation. Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 was utilized to edit cfGnRH and LH genes through microinjection.

Targeted cfGnRH and FSH gene mutagenesis were found in 52.9% and 63.2% P_1 fish respectively through TALEN technology. Fish spawning, reproductive behavior and the hatching of their eggs were suppressed. Luteinizing hormone-releasing hormone analog (LHRHa) hormone therapy resulted in good spawning and hatch rates for mutants, which were not significantly different from controls ($P>0.05$). Human chorionic gonadotropin (HCG) hormone therapy resulted in spawning rates of 80% for female mutants and 88.9% for male mutants, and mean hatch rate of 35.0% for F_1 embryo, which was not significantly different from controls ($P>0.05$).

LH gene mutagenesis was found around 60 bp upstream of the expected site in 38.9% P_1 fish through ZFN technology. None of the female mutant fish could spawn without hormone therapy. P_1 mutant males had lower spawning rate than controls with egg hatch rates of 1%. HCG hormone therapy resulted in good spawning and hatch rates for mutants, which were not significantly different from controls ($P>0.05$). No obvious effects on other economically important traits were observed after the knockout of reproductive genes in all P_1 and F_1 mutants using TALEN and ZFN technologies through double electroporation.

CRISPR/Cas9 induced 50% mutagenesis at the targeted sites of cfGnRH and LH gene respectively. No obvious pleiotropic effects were observed in terms of body weight. CRISPR-Cas9 nucleases enabled higher efficient genome editing than ZFNs, similar efficient genome editing than TALENs, however with low hatchability through microinjection.

Gene editing of channel catfish for reproductive confinement of gene-engineered, domestic and invasive fish to prevent gene flow into the natural environment appears promising.

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List of Abbreviations

cfGnRH	Catfish type gonadotropin releasing hormone
FSH	Follicle stimulating hormone
LH	Luteinizing hormone
ZFN	Zinc finger nuclease
TALEN	Transcription activator-like effector nuclease
CRISPR	Clustered regularly interspaced short palindromic repeats
LHRHa	Luteinizing hormone releasing hormone analogue
HCG	Human chorionic gonadotropin

Ⅰ Gene Editing of the Catfish Gonadotropin Releasing Hormone Gene and Hormone Therapy to Control the Reproduction in Channel Catfish, *Ictalurus punctatus*

Abstract

Transcription activator-like effector nucleases (TALENs) plasmids targeting the catfish gonadotropin-releasing hormone (cfGnRH) gene were delivered into fertilized eggs with double electroporation to sterilize channel catfish (*Ictalurus punctatus*). Targeted cfGnRH gene mutagenesis was achieved in 52.9% of P_1 fish. P_1 mutants had lower spawning rates (20.0-50.0%) when there was no hormone therapy when compared to the control pairs (66.7%) as well as having lower average egg hatch rates (2.0% versus 32.3%-74.3%) except for one cfGnRH mutated female that had a 66.0% hatch rate. After establishing low fertility in 2016, luteinizing hormone-releasing hormone analog (LHRHa) hormone therapy resulted in good spawning and hatch rates for mutants in 2017, which were not significantly different from controls (P>0.05). No exogenous DNA fragments were detected in the genome of mutant P_1 fish, indicating no integration of the plasmids. No obvious effects on other economically important traits were observed after the knockout of reproductive genes in the P_1 and F_1 mutant fish. Growth rates, survival, and appearance between mutant and control individuals were not different. Complete knock-out of reproductive output was not achieved as these were mosaic P_1 brood stock.

1 Introduction

Channel catfish (*Ictalurus punctatus*) and their hybrids account for more than 60% of aquaculture production in US (Liu et al. 2016). The catfish industry is being severely challenged during recent years as a result of higher operating costs, disease, and competition from cheaper imported frozen catfish (Hanson and Sites 2014).

A potential future tool to help address this problem is the utilization of genetically engineered catfish. Transgenic fish can result in increased yield (Martínez et al. 2000), disease resistance (Chiou et al. 2014), survival ability in extreme environments (Chen et al. 2015) and the secretion of specific proteins (Dunham et al. 2006). However, concerns have been expressed regarding the potential ecological and genetic effects of these fish (Hedtrick 2001). An effective fish sterilization technology could prevent environmental risk of transgenic fish.

Methods often used to genetically sterilize fish include triploid induction, genetically monosex populations and interspecific hybridization. However, these techniques do not always result in 100% sterility, and they require fertile diploid brood stock, thus environmental risk cannot be eliminated. Additionally, triploidy is a labor-intensive procedure. Commercial scale triploidy in *ictalurid* catfish would be labor intensive, and triploidy usually decreases the overall performance of fish (Dunham 2011). Transgenic sterilization technology has been developed to knock down genes essential for reproduction (Uzbekova et al. 2000). However, transgenesis would bring in possible adverse pleiotropic effects, including viral components (Thresher et al. 2009), unwanted transgenesis (Wong and Van Eenennaam 2008) and reduced growth rate (Li et al. 2017, 2018).

Targeted genome editing technologies, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced

short palindromic repeats (CRISPR), can be utilized to mutate reproductive genes to sterilize catfish to prevent the potential risks of genetically modified fish. ZFNs was the earliest gene editing technology, utilizing a FokI endonuclease domain and a zinc finger deoxyribonucleic acid (DNA) binding domain. Compared to ZFNs, TALENs can be expeditious and more easily designed using a simple 'protein-DNA code' that relates modular DNA-binding transcription-like (TAL) repeat domains to individual's bases in a target-binding site (Joung and Sander 2013). Similar to ZFNs, TALENs are composed of a FokI endonuclease domain and a TAL effector domain, which can induce double-strand breaks (DSBs) to specific DNA sites (Baker 2012). Usually DSBs are repaired by non-homologous end joining (NHEJ) and typically result in small insertions or deletions that will disable the gene because of the change or shift of the translational reading frame (Stoddard 2011). ZFN technology has been used to knock out the luteinizing hormone (LH) gene to effectively create gene-edited sterile channel catfish (Qin 2015, Qin et al. 2016).

TALEN-mediated mutations have been reported in various animals, including worm (*Bombyx mori*) (Takasu 2016), mosquito (*Aedes aegypti*) (Basu et al. 2015), frog (*Xenopus tropicalis*) (Lei et al. 2012), ascidian (*Ciona intestinalis*) (Treen et al. 2014), zebrafish (*Danio rerio*) (Huang et al. 2011, Liu et al. 2016), medaka (*Oryzias latipes*) (Chiang 2016) and yellow catfish (*Tachysurus fulvidraco*) (Dong et al. 2011). TALENs have been used to knock out LH gene and follicle stimulating hormone (FSH) genes to inhibit gonadal development and ovulation in zebrafish (*Xenopus tropicalis*) (Zhang et al. 2015).

Gonadal maturation in teleost fishes is principally controlled by hypothalamus-pituitary-gonad (HPG) axis. Gonadotropin-releasing hormone (GnRH) plays a pivotal role to adjust the differentiation of the gonad via the HPG axis (Schaefer et al. 2016, Yaron et al. 2003). GnRH is

a releasing hormone secreted from the hypothalamus by neurosecretory cells. In teleost fishes, GnRH-expressing neurons are distributed among two or three distinct GnRH populations within the brain (Anderson et al. 2001, Choi et al. 2016). Those two or three types of GnRH gene are all composed of three introns and four exons (Kitahashi et al. 2005, Suzuki et al. 2000). GnRH can stimulate the synthesis and release of pituitary gonadotropin, followed by stimulating the secretion of steroid hormones (Sherwood et al. 1993). Additionally, GnRH might be involved in regulating the reproductive behavior of fish (Temple et al. 2003). GnRH can activate the secretion of other pituitary hormones, which include growth hormone (Klausen et al. 2001), prolactin and somatolactin (Weber et al. 1997). Siluriformes possess two forms of GnRH — chicken GnRH-II (cGnRH-II) and cfGnRH. The former is commonly accepted as a neuromodulator playing a role in sexual behavior, while the latter is the hypothalamic GnRH form and considered responsible for the release of gonadotropin, playing a critical role in sexual maturation in catfish (*Clarias gariepinus*) (Blomenröhr et al. 2002, Dubois et al. 2001, Kumar et al. 2005).

All vertebrate groups share a common structure of GnRH with three introns and four exons. The typical structure of the GnRH precursor protein consists of an untranslated region and a signal peptide of about 23 amino acids, a decapeptide followed by a 3-amino acid cleavage site, and a GnRH-associated peptide (GAP) followed by C-terminal region of total about 60 amino acids (Zmora et al. 2002). Nikolics et al. (1985) reported that a complete GAP peptide has dual activities, one inhibits the activity of prolactin and the other stimulates the releasing of gonadotropin-releasing in cultured rat pituitary cells. Millar et al. (1986) stated that GAP could stimulate the release of gonadotropins (LH and FSH) *in vitro.* Andersen and Klunglan (1993) reviewed the function of GAP and GnRH and concluded that GAP might play a role in

conferring the structure and stability of prepro-GnRH. Wetsel and Srinivasan (2002) proposed that GAP serves as a linker to connect the decapeptide by the amide donor glycine and the dibasic residues lysine and arginine so that pro-GnRH can be guided and routed out of the endoplasmic reticulum into the secretory pathway. A complete amino acid constitution of GAP peptide is required to exert full activity in conjugate with GnRH decapeptide.

GnRH is responsible for the release of gonadotropins, FSH and LH. FSH and LH vitalize germ cell development via activation of the FSH receptor and LH receptor, partly by stimulating the production of sex steroids in gonadal somatic cells and by releasing gonadal paracrine growth factors that manage the development of germ cells (Taranger et al. 2010). These gonadal sex steroids and growth factors consecutively provide positive or negative feedback to the brain and pituitary to modulate FSH and LH production and secretion to complete the HPG axis (Zohar et al. 2010). FSH plays a major role in the earlier stage of the reproductive cycle by activating steroidogenesis, vitellogenesis and spermatogenesis; while LH plays a vital role in the later stage on stimulating spermiation, ovulation and maturation of oocytes (Kumar et al. 2001, Ogiwara et al. 2013, Swanson et al. 2003).

The objectives of this study were to use TALENs technology to block the function of cfGnRH gene on the genomic level, leading to sterility in channel catfish, and then restore the fertility of gene edited sterile channel catfish through hormone therapy. Specifically, TALENs plasmids targeting the cfGnRH gene were delivered into eggs and fertilized eggs with double electroporation technique. Fish mating experiments were utilized to evaluate the reproductive capability of mutant catfish. LHRHa was applied to reinstate the fertility of gene edited sterile channel catfish. Mutation analyses were employed on F_1 fish to verify the heredity of mutations.

Hatch rate, survival rate and body weight were measured in F_1 fish to assess the pleiotropic effects.

2 Materials and Methods

2.1 Construction of Plasmid

Transcription activator-like effector nucleases (TALENs) are proteins selected from the bacteria *Xanthamonas*. *Xanthomonas* transcription activator-like effector nucleases (XTNTMs) plasmids were designed and assembled by Transposagen Company (Lexington, KY). Each XTN was cloned into the SQT281 vector in the TAL repeat region and driven by the cytomegalovirus (CMV) promoter, T7 promoter, then followed by FokI nuclease domain (Fig. 1). The TALENs target sites were designed to be located at exon3, which is a GnRH-associated peptide (GAP) region of cfGnRH gene (GenBank Accession No. XM_017468372.1). GAP can stimulate the release of LH and FSH, and suppressed the release of prolactin (Thomas et al. 1988, Yu et al. 1988). Each TALEN comprised an 18 bp (5'-TTCACCTCGGAATAAACT-3') left DNA binding site, or an 18 bp (5'-TGAGCTGTGCACCAGCAG-3') right DNA binding site.

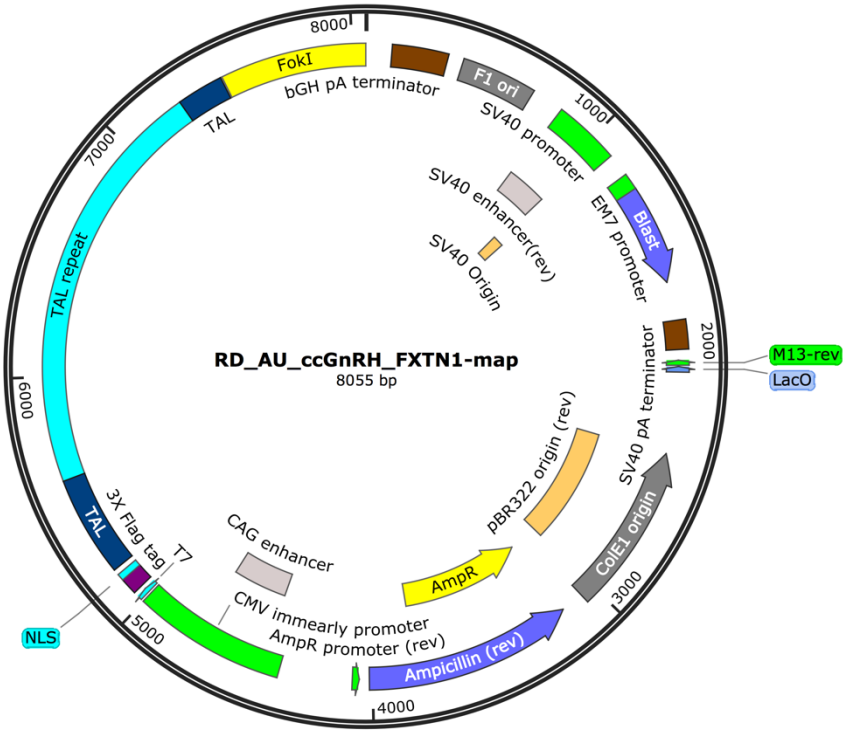


Figure 1. Schematic representation of transcription activator-like effector nucleases (TALENs) plasmid structure targeting catfish type gonadotropin releasing hormone (cfGnRH) gene of channel catfish (*Ictalurus punctatus*). *CMV prmt* cytomegalovirus promoter, *NLS* nuclear localization signal, *BGH pA* bovine growth hormone and polyadenylation signal, *AmpR* ampicillin resistance gene.

2.2 TALEN Plasmid Replication, Extraction and Dilution

The TALEN plasmids were transformed into One Shot® TOP10F′ Competent Cells (Invitrogen, Grand Island, NY), following the transformation procedures from the manufacturer. One hundred µL of each transformation mix vial was spread on the LB agar plate with 100 µg ml^{-1} ampicillin separately. A single colony was picked from each plate and then cultured in 400 mL LB broth with 100 µg ml^{-1} ampicillin separately. Then the TALEN plasmids were extracted with the IsoPure Plasmid Maxi II Prep Kit (Denville, Holliston, MA). DNA agarose gel

electrophoresis and spectrophotometry were then used to test the quantity and quality of the TALEN plasmids. Equal amounts of both left and right TALEN plasmids were mixed together. They were diluted with 2 mL saline (0.9% NaCl) to obtain a 25 μg ml^{-1} mix solution and diluted with 5 mL TE buffer (5mM Tris-HCl, 0.5M EDTA, pH=8.0) to collect a mix solution with the same concentration, respectively.

2.3 Experimental Brood Stock and Gametes

Sexually mature females of Kansas Random strain of channel catfish (*Ictalurus punctatus*) were selected and implanted with luteinizing hormone releasing hormone analog (LHRHa) (Reproboost® Implants, Center of Marine Biotechnology, Columbus Center, Maryland, USA) with a dose of 90 μg kg^{-1}. All showed outstanding secondary sexual characteristics. Eggs of two anesthetized ovulating females were hand stripped into dried stainless steel pie pans greased with vegetable shortening. Two-hundred eggs were collected for each experimental group and control group. One Kansas Random strain male and one Auburn-Rio Grande strain male (Dunham and Smitherman, 1983) were euthanized. Mixed testes were placed into a mesh strainer and macerated manually into saline (0.9% NaCl) to collect sperm.

2.4 Fertilization, Electroporation and Incubation

The first electroporation was conducted on the collected sperm homogenate with the TALEN saline solution using a Baekon 2000 macromolecule transfer system (Baekon, Inc., Saratoga, CA) with parameters of 6kV, 27 pulses, 0.8s burst, 4 cycles and 160μs (Su et al. 2014). Then two hundred prepared eggs were artificially fertilized with the electroporated sperm. After one hour, the embryos were gathered and immersed with the TALEN TE buffer solution for ten

minutes. Then the second electroporation was conducted with the same procedure. The control group was generated following the same protocols, but with the absence of TALEN plasmids. Embryos were then moved into 10 L tubs with Holtfreter's solution containing 10 ppm doxycycline and incubated statically until hatch. Dead embryos were removed daily before Holtfreter's solution was changed.

2.5 Sample Collection and Mutation Analysis for P_1 generation

Embryos were cultured for 6-7 days until hatch in tubs at 27°C and then transferred into a recirculating system. After 6 months, the genomic DNA was extracted from the pelvic fin and barbel samples. Samples were digested with cell lysis buffer and proteinase K following the protocols by Cheng et al. (2014). DNA agarose gel electrophoresis and spectrophotometry were then used to test the quantity and quality of the genomic DNA. Then Roche Expand High FidelityPlus PCR System (Roche, Indianapolis, IN) was used to amplify the channel catfish cfGnRH gene on these DNA samples. The following primers were utilized: forward sequence 5'–ATGGATGCTGTCTTTGTTTTCC-3'; reverse sequence 5'–CCACACGAAATAAAGG CAAAG-3' (Table 1), and the amplification procedure was: initial denaturation for 2 min at 94°C, followed by 29 cycles of 94°C for 30s, 60°C for 30s and 72°C for 40s, and the final elongation for 10min at 72°C.

Gene mutations were detected using Surveyor® Mutation Detection Kit for Standard Gel Electrophoresis (Integrated DNA Technologies, Coralville, IA) (Qin et al. 2015). The key component of the kit is Surveyor Nuclease, a member of the CEL family of mismatch-specific nucleases derived from celery, which can be used to perform a highly specific cleavage on the 3' side near the mutation site of the two strands of the heteroduplex DNA. The recognized mutant

forms include single base or multiple base insertions, deletions or mismatches (Qiu et al. 2004). Surveyor Nuclease has been a powerful and repeatable tool for mutation testing. It has been used to detect mutations and polymorphism in human, mammal, bacteria, and plant genomes (Pimkin et al. 2007; Tsuji and Niida, 2008). The processes were as follows: first, the PCR products was hybridized using a PCR machine to form a heteroduplex using a thermal cycler as 95°C 10min, 85°C 1min Ramp 2°C/s, 75°C 1min Ramp 0.3°C/s, 65°C 1min Ramp 0.3°C/s, 55°C 1min Ramp 0.3°C/s, 45°C 1min Ramp 0.3°C/s, 35°C 1min Ramp 0.3°C/s and 25°C1 min Ramp 0.3°C/s; then a nuclease reaction system, which included DNA hybridization system, 150mM $MgCl_2$, 5×PCR buffer, Surveyor enhancers S and Surveyor nucleases were established and incubated in a PCR machine under 42°C for 1 hour; finally, the 2% UltraPure Agrose-1000 (Invitrogen, Grand Island, NY, USA) was used to perform electrophoresis to test the mutation.

2.6 TA Clone and Sequencing

To confirm the presence of the mutations, Roche high fidelity PCR amplification targeted cfGnRH gene was performed with the sample DNA. Then TOPO® TA Cloning® kit was used to set up a 6μL TOPO cloning system, which included 4μL amplified DNA product, 1μL salt solution and 1μL TOPO vector. The system was mixed and incubated under room temperature (22-23°C) for 5min, followed by competent cell transfection. First, 2μL of the cloning system was added into one vial of One Shot®TOP10F'competent cells and put on the ice for 30min. Then 42°C heat shock was implemented using PCR machine for 30s. After that, the tube was placed on ice immediately. The next, 250μL LB (Tryptone 10g/L, Yeast extract 5g/L, NaCl 10g/L, Agarose 10g) broth was mixed together in a 1.5ml tube and placed in the 37°C shaker and shaken at 200rpm for 1 hour. Next, 50μL, 80μL, and 100μL mixtures were spread on the LB

(Tryptone 10g/L, Yeast extract 5g/L, NaC l10g/L, Agarose 10g) medium and placed in the 37°C incubator overnight. Ten monoclonal colonies were picked from each plate, mixed with 400ml LB (Tryptone 10g/L, Yeast extract5g/L, NaCl 10g/L, Agarose10g) broth and placed in the 37°C incubator overnight. The amplified bacterial solution was dispensed into a 96-wells plate and sent to the Eurofins Genomics Company (Louisville, KY, USA) for sequencing. Finally, sequencing results were analyzed using T-Coffee tool (Notredame et al. 2000).

2.7 Plasmid Integration Inspection

PCR was conducted to determine if plasmids were integrated into the genome or persisted in the cytoplasm. Two pairs of primers targeting cfGnRH gene were designed to detect the plasmid DNA in mutant fish. The amplification regions were CMV promoter region and TAL repeats region respectively. They were amplified using the primers as shown in Table 1. The amplification procedure was as follows: initial denaturation for 2 min at 94°C, followed by 29 cycles of 94°C for 30s, 60°C for 30s and 72°C for 40s, and the final elongation for 10min at 72°C. The results were generated using electrophoresis.

Table 1. Primer sequences, annealing temperature used for the amplification and the product length of catfish type gonadotropin releasing hormone (cfGnRH), cytomegalovirus (CMV) promoter and transcription-like (TAL) repeats regions in channel catfish (*Ictalurus punctatus*).

Targeting Site	Strand	Sequence	Annealing temperature	Product length (bp)
cfGnRH	Forward	5'-ATGGATGCTGTCTTTGTTTTCC-3'	60 ℃	550
	Reverse	5'-CCACACGAAATAAAGGCAAAG-3'		
CMV promoter	Forward	5'-AACAACAACGGCGGTAAG-3'	60 ℃	114
	Reverse	5'-CCCATTATTGTTCGCGATTG-3'		
TAL Repeats	Forward	5'-GCATGACGGAGGGAAAC-3'	60 ℃	215
	Reverse	5'-CCATTATTGTTCGCGATTGA-3'		

2.8 Reproductive Evaluation

The fingerlings were transferred to 0.04- hectare pond or a recirculating system when they reached 15-20cm for further growth and maturation. Mating experiments began on June 5, 2016. There were 8 mutant (M) males and 5 mutant females selected to mate with wild type (Wt) females and males (Wt♀×M♂, M♀×Wt♂). There were five pairs of control of Kansas Random strain of channel catfish selected for natural spawning without hormone injection. These fish were paired in 48cm×36cm×21cm aquaria with constant water flow and compressed air for aeration. All fish showed outstanding secondary sexual characteristics. The sexually mature males had well-developed head muscles and dark mottling in the lower jaw and abdomen. The sexually mature females exhibited fuller, rounder abdomen and reddening genital area compared to immature females.

For testing the fertility of mutant males, only the paired wild type females were implanted with 75μg/kg LHRHa for induced spawning. Then the wild type females that did not spawn within 5 days were implanted again with 70μg/kg LHRHa. If the females did not spawn

after two implantings, they were replaced by other wild type females and the hormone implantation for laying eggs. For testing the fertility of mutant females, the pairs were given 12 days to spawn naturally with the wild type males and without hormone injection.

2.9 Hormone Therapy

In 2016, 3 mutant males and 3 mutant females mated with each other and implanted with 90μg/kg LHRHa (M♀×M♂). After 9 days, all these three pairs without spawning were implanted with 90μg/kg LHRHa again.

After establishing infertility in 2016, hormone therapy was conducted in 2017. There were 5 mutant males and 6 mutant females with outstanding secondary sexual characteristics selected to mate with each other (M♀×M♂). First, both females and males were injected with 100μg/kg LHRHa solution. If females and males did not spawn within 5 days, they were injected again with 80μg/kg LHRHa. Five pairs of control of Kansas Random strain of channel catfish were selected for natural spawning without hormone injection.

2.10 F_1 Fish Culture

When egg masses were obtained, they were placed in baskets in hatching troughs with constant water flow and aeration. Calcium chloride solution was continually dripped into the trough to ensure 40-50ppm hardness (Steeby, 2005). Eggs were gently agitated with a paddlewheel beginning 2 hours after spawn collection. The egg masses were prophylactically treated with 100ppm formalin or 32ppm copper sulfate every 8 hours to avoid fungus (Small et al. 2006). The treatments were terminated 12 hours before hatch.

Catfish embryos began hatching in 7 days with a water temperature between 26-28℃.

They consumed their yolk sac and began swim up stage 3 days post hatching. They were then fed artemia (Brine Shrimp Eggs, Carolina Biological) three times a day and stocked into a recirculating system with densities of 1000 fish per 90L tank and 100 fish per 60L aquaria of each family. After one month fry were fed Purina® AquaMax® Fry Powder (Purina, St. Louis, MO) three times a day. As fry grew they were then fed Purina® AquaMax® Fry Starter 100. After further growth fry were moved to 90L tank at a stocking density of 500 fish per tank and fed Purina® AquaMax® Fry Starter 200 and 300 three times a day.

2.11 Sample Collection and Mutation Analysis for F_1 generation

When 2016 spawned F_1 fish were 1.5-years-old, ventral fin samples were collected from 30 fish in each family. When 2017 spawned F_1 fish were 1-year-old, ventral fin samples were collected from 30-100 fish in each family. Surveyor mutation analysis was conducted. The procedures were the same as described above.

2.12 Pleiotropic Effects

For P_1 fish, survival rate for embryos, 6-month-old fingerlings, 3-year-old and 4-year-old adult fish was measured. For F_1 fish, survival rate and body weight for F_1 1-year-old and 1.5-year-old fish were measured. Dead fish from a columnaris outbreak in one tank were collected. The reproductive behavior was observed through the bottom of overhead aquariums.

2.13 Statistical Analysis

Statistical analysis of mutation rate, survival rate, hatch rate and body weight were performed with R studio software (RStudio Inc, Boston, MA). Mutation rate, survival rate, and

hatch rate were compared utilizing Fisher's exact test in case of small sample size. Student's *t*-test was performed to compare hatch rate and spawning rate between treatments and controls for P_1 fish. Student's *t*-test was also applied to compare survival rate and mutation rate between F_1 families before and after hormone therapy, and compare body weight in each F_1 families. Shapiro-Wilk's test was used to test the normality of the data. Comparisons of statistical significance were set at $P<0.05$, and all data were presented as the mean ± standard error (*SEM*).

2.14 Sequence Structure Analysis

Catfish type of GnRH mRNA sequences were retrieved from the nucleotide NCBI database (https://www.ncbi.nlm.nih.gov/). Amino acid sequences of cfGnRH protein were translated using ExPASy software (https://web.expasy.org/translate/). Amino acid sequences of GAP was verified in the study from Lamkom (2010). Sequences of cfGnRH protein and GAP were used as queries to BLAST against protein data bank (PDB) database with a cutoff E-value of $1e\text{-}^{10}$. If it shows significant similarity with query sequence, then the secondary structures of cfGnRH protein and GAP were predicted using PDBsum software (http://www.ebi.ac.uk/thornton-srv/databases/cgibin/pdbsum/GetPage.pl? pdbcode=index.html). If it doesn't show significant similarity with query sequence, then the secondary structures of cfGnRH gene and GAP were predicted using Pole Bioinformatique Lyonnais (PBIL) server (https://prabi.ibcp.fr/htm/site/web/home). The three-dimensional protein structure was predicted using the I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). Only models with the C-score between 2 and -4 were considered. C-score is a confidence score for estimating the quality of predicted models. It is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is

typically in the range of [-5, 2], where a C-score of higher value signifies a model with a high confidence and vice-versa. The visualization of predicted three-dimensional structures was performed using the Jmol software. The predicted model was downloaded from I-TASSER as a PDB file. The predicted secondary protein structure based on the predicted 3D model was displayed using the PDBsum server and compared with the result from PBIL.

3 Results

3.1 Analysis of Mutation Efficiency and Plasmid Integration of P_1 Generation

The cfGnRH gene was successfully mutated in 27 of 51 6-month-old P_1 fingerlings with a 52.9% mutation rate. The surveyor mutation assay results of pelvic fin and barbel tissues showed three or five bands on gel. The 350bp band indicated the cfGnRH gene was cut at the expected site (Fig. 2A).

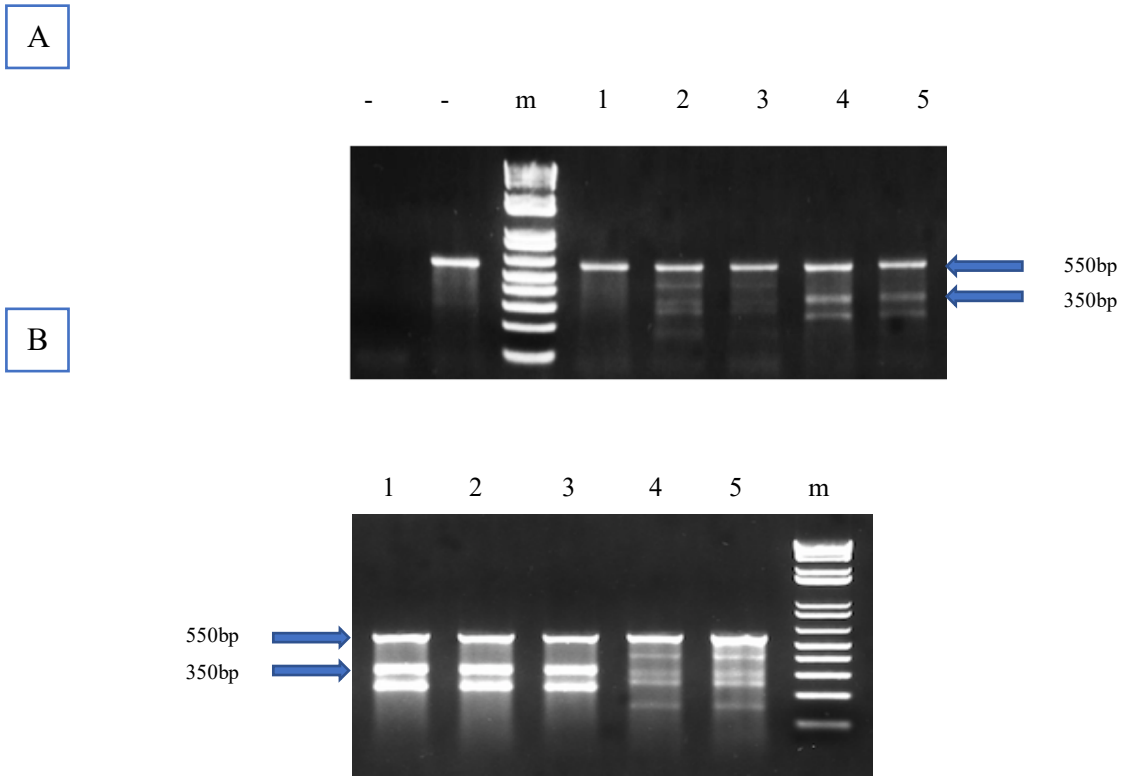


Figure 2. Identification of edited catfish type gonadotropin-releasing hormone (cfGnRH) gene in P_1 and F_1 channel catfish (*Ictalurus punctatus*) using the Surveyor mutation detection assay. **(A)** The left "-" indicates the negative control without template. The right "-" indicates the negative control with wild type template; "m" indicates 1kb DNA ladder; 2, 3, 4 and 5 are channel catfish with mutation; Lane 1 is a channel catfish without the mutation. **(B)** The negative control is not shown in this image. "m" indicates 1kb DNA ladder; 1, 2,3, 4 and 5 are channel catfish with mutation.

Sequencing of cloned PCR products confirmed that the channel catfish were successfully mutated for the cfGnRH gene and included insertions, deletions, and substitutions, all of which within the target TALEN cutting site. These mutations were in the GAP region of the cfGnRH gene (Fig. 3A and Fig. 3B).

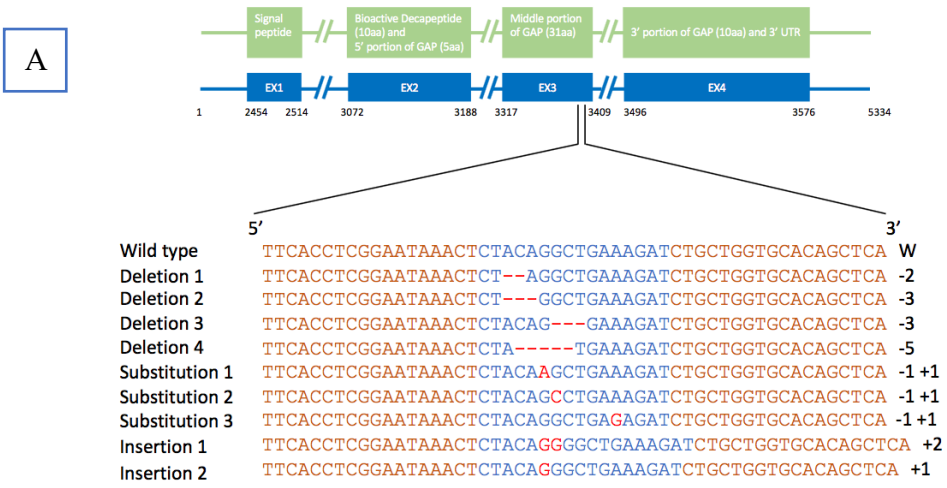


Figure 3. Nucleic acid sequences and corresponding predicted amino acid sequences of catfish type gonadotropin-releasing hormone (cfGnRH) gene in wild type channel catfish (*Ictalurus punctatus*) and after edited with transcription activator-like effector nucleases (TALENs). **(A)** EX1-4 indicates exon 1-4. The wild-type channel catfish cfGnRH gene sequence is shown on the top. Sequences in orange are the target binding sites of the TALENs. Sequences in blue on the middle portion of gonadotropin-releasing hormone associated peptide (GAP) are the expected cleavage sites of the nucleases. Red dashes and letters indicate the deletion/insertion/substitution of nucleotides. Numbers at the end of the sequences show the number of nucleotides deleted (-) or inserted (+) in the edited cfGnRH gene. **(B)** Predicted amino acid sequences with incomplete domain were due to frame-shift reading, resulting in a premature stop (red color) codon. Single amino acid substitutions or deletions (red color) were due to single nucleotide substitution mutation or three nucleotides deletion.

Neither CMV promoter region nor TAL repeats region were detected via PCR for any TALEN-cfGnRH mutant fish. Thus, none of the fingerlings carried the exogenous DNA (Fig. 4A and Fig. 4B).

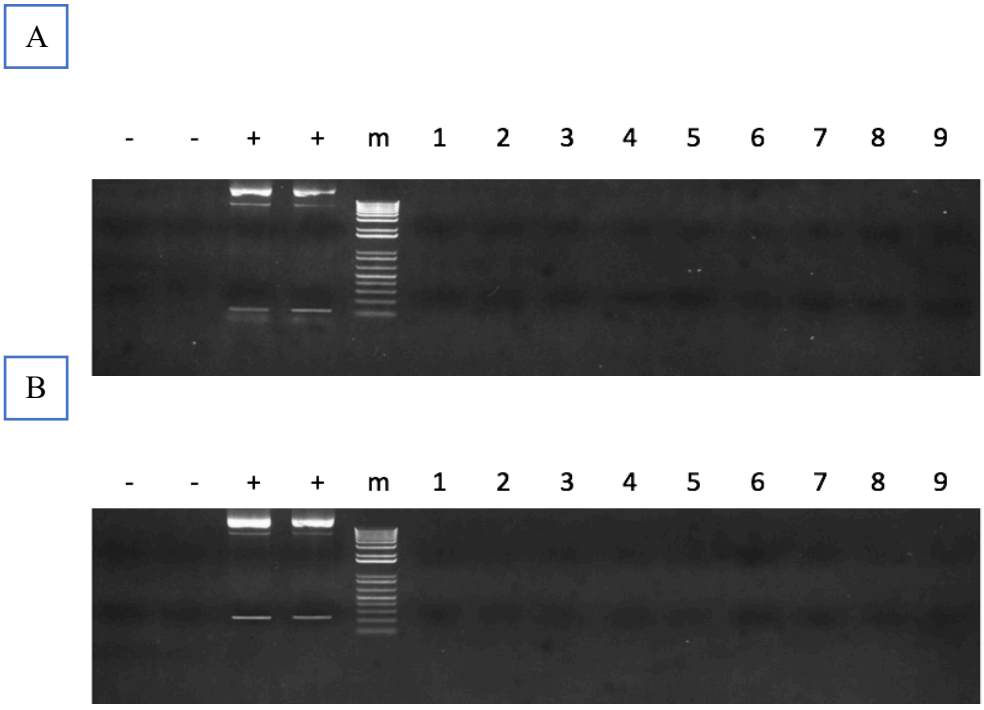


Figure 4. Polymerase chain reaction (PCR) inspection of potential transcription activator-like effector nucleases (TALENs) plasmid integration into channel catfish (*Ictalurus punctatus*) genome. In **(A)** and **(B)**, the left lanes of "-"indicate the negative controls without template while the right lanes of "-"indicate the negative controls with wild-type channel catfish DNA as a template; "+" indicates the positive controls with left and right TALEN plasmids as a template, respectively; "m" indicates 1 kb DNA ladders. Numbers represent samples from channel catfish individuals carrying mutated cfGnRH gene; the same number indicates the same individual in **(A)** and **(B)**. **(A)** and **(B)** represent the PCR detection with different specific primers designed to amplify the cytomegalovirus (CMV) promoter region and the transcription activator-like (TAL) repeats region, respectively.

3.2 Spawning Experiments and Hormone Therapy

In 2016, 3 of 5 (60.0%) control pairs spawned with a 74.3% average hatch rate without LHRHa implantation. P_1 mutants had spawning rates of 20% for females and 50% for males when mated with wild type males and females, respectively and when there was no hormone therapy with mean egg hatch rates of 2.0% except for one cfGnRH mutated female that had a 66.0% hatch rate (Table 2). The mean hatch rate of the mutant group was lower than the control group (P=0.009). Three pairs of mutant channel catfish did not spawn after LHRHa hormone therapy with a dose of 90 μg/kg, however, it was late in the spawning season.

After establishing low fertility for GnRH gene edited channel catfish in 2016, LHRHa hormone therapy of 100 μg/kg was implemented in 2017. Five of seven (71.4%) control pairs spawned with a 32.3% average hatch rate without LHRHa injection, while 2 of 3 (66.7%) control pairs spawned with a 56.5% average hatch rate with LHRHa injection in 2017. Spawning rate for female and male mutants mated together and receiving hormone therapy were 50.0% and 60.0% (one male spawned with two different females), respectively. The spawning rates of mutants were not different (P=0.923) from controls without LHRHa injection, 71.4%, and controls with LHRHa injection, 66.7%. F_1 embryo hatch rate for female and male mutants that received

hormone therapy were both 72.1%, which was not different (P=0.728, P=0.615, respectively) from non-injected control and injected control, 32.3% without LHRHa injection and 56.5% with LHRHa injection (Table 2).

Table 2. The spawning rate of P_1 mutants and the mean embryo hatch rate of F_1 channel catfish (*Ictalurus punctatus*) before and after 90 μg/kg luteinizing hormone-releasing hormone analog (LHRHa) hormone therapy in 2016, and after 100 μg/kg LHRHa hormone therapy in 2017. Two types of controls were used, including non-injected control (nCTRL) and injected control (iCTRL) with 90 μg/kg LHRHa solution. In 2016, 5 mutant females and 8 mutant males were paired with wild type fish respectively before hormone therapy. Only the wild type females were implanted with 75 μg/kg LHRHa for induced spawning. In 2016, 3 mutant females and 3 mutant males were paired with each other with 90 μg/kg LHRHa hormone therapy. In 2017, 6 mutant females were paired with 5 mutant males with 100 μg/kg LHRHa hormone therapy (one male had two consecutive females). Hatch rate is the number of live embryos divided by the total number of embryos in each family and multiplied by 100. Mean hatch rate data were presented as the mean ± standard error (*SEM*).

Treatment	Sex	Spawning rate and the mean of the embryo hatch rate											
		Before hormone therapy (2016)				After hormone therapy (2016)				After hormone therapy (2017)			
		Fish N	Spawned fish N	Spawning rate (%)	Mean hatch rate ± SEM (%)	Fish N	Spawned fish N	Spawning rate (%)	Mean hatch rate (%)	Fish N	Spawned fish N	Spawning rate (%)	Mean hatch rate (%)
cfGnRH	F	5	1	20.0	66.0	3	0	0	/	6	3	50.0	72.1± 0.07
	M	8	4	50.0	2.0± 0.01	3	0	0	/	5	3	60.0	72.1± 0.07
nCTRL	F and M	5	3	60.0	74.3± 0.02	/	/	/	/	7	5	71.4	32.3± 0.20
iCTRL	F and M	/	/	/	/	/	/	/	/	3	2	66.7	56.5± 0.34

Notes:
Hatch rate was significantly different between cfGnRH gene-edited group and nCTRL group before hormone therapy in 2016 (Student's t test, P=0.009).
Hatch rates were not significantly different between cfGnRH gene-edited group and nCTRL group, and between cfGnRH gene-edited group and iCTRL group after hormone therapy in 2017 (Student's t test, P=0.728, P=0.615).
Spawning rates were not significantly different among mutant females, mutant males, and control before hormone therapy in 2016 (Fisher's exact test, P=0.565).
Spawning rates were not significantly different among mutant females, mutant males, and nCTRL and iCTRL after hormone therapy in 2017 (Fisher's exact test, P=0.923).
Three pairs in 2016 did not spawn with hormone therapy likely due to the lateness of the spawning season and perhaps over ripeness.

3.3 Analysis of Mutation Efficiency of F_1 Generation

Four F_1 families were generated from mutant (M) fish and wild type (Wt) fish in 2016, including 2016 GnRH-1 (Wt♀×M♂), 2016 GnRH-2 (Wt♀×M♂), 2016 GnRH-3 (Wt♀×M♂) and 2016 GnRH-4 (M♀×Wt♂). Three M♀×M♂ F_1 families were produced in 2017, including 2017 GnRH-1, 2017 GnRH-2, and 2017 GnRH-3. The Surveyor mutation detection assay results for mutated fish showed three or five bands on the gel. The 350bp band indicated the cfGnRH gene was cut at the expected site (Fig. 2B). The 1.5-year-old 2016 F_1 families had 60.0%, 63.3%, 76.6%, 66.7% inheritance of the mutations, respectively. The 1-year-old 2017 F_1 offspring families had 53.0%, 46.7%, 51.0% inheritance of the mutations, respectively (Table 4).

3.4 Pleiotropic Effects

For P_1 fish in the TALENs treatment group, 200 eggs were double electroporated, 76 of the 200 hatched with a 38.0% hatch rate. After 6 months, the fry survival rate of the TALEN cfGnRH group was 67.1%. For the control group, 84 of 200 eggs were double electroporated without TALEN plasmids with a 42.0% hatch rate. After 6 months, the fry survival rate was 75.0%. No significant differences were found between treatment group and control group for both hatch rate and survival rate (P=0.475, P=0.223). After three years, survival rates were 50.0% for mutant fish and 33.3% for non-mutant full-sibling fish. After four years, survival rates were 41.2% for mutant fish and 37.5% for non-mutant full-sibling fish in a recirculating system; while in the pond, the survival rates were 46.9% for mutant fish and 33.3% for non-mutant fish. No significant differences were found between mutants and non-mutants for 3-year-old P_1 and 4-year-old P_1 in the recirculating system and pond, respectively (P=0.270, P=1.000, P=0.672)

(Table 3). Although, the means were not different, the gene-edited fish had a consistently higher observed survival.

During courtship, sexually mature channel catfish males and females usually use their tail fins to cover each other's eyes, and then their bodies quiver. The female deposits eggs into the water to form an egg mass, and the male discharges sperm almost immediately to fertilize the eggs. However, in this case, four of eight mutant males and four of five mutant females did not exhibit these normal reproductive behaviors, although all fish exhibited outstanding secondary sexual characteristics. In 2016, all the wild type females were crossed with mutant males, but abnormal courtship for mutant males was observed through the bottom of the transparent aquaria. The mutant males were less willing to get closed to the females. And they didn't use their tail fins to cover females' eyes or quiver their bodies. Some wild type females laid immature eggs without generating an egg mass. LHRHa hormone therapy resulted in good spawning and hatch rates for mutants in 2017. Three of five (60.0%) mutant males and three of six (50.0%) mutant females exhibited normal reproductive behavior. Their spawning rate and hatch rate were not significantly different from controls (P=0.923).

For F_1 fish, four families were obtained in 2016 before LHRHa hormone therapy, three families were obtained in 2017 after 100 μg/kg LHRHa hormone therapy. The survival rates of the families obtained before hormone therapy with a mean of 53.3% were significantly lower than the families obtained after hormone therapy with a mean of 86.2% (P=0.014). The mutation rates of the families obtained before hormone therapy with a mean of 66.7% were significantly higher than the families obtained after hormone therapy with a mean of 50.2% (P=0.013) (Table 4). No significant difference in body weight between mutant fish and non-mutant fish were found in each family (P=0.826, P=0.598, P=0.749, P=0.296, P=0.321, P=0.563, P=0.927). After

evaluating the body weight in each family, an acute columnaris (*Flavobacterium columnare*) infection occurred in one family, 2017 GnRH-1. The mutation rate for this family was 53.0% before the infection. Fifty-six dead fish were collected in one tank, 36 of 56 (64.3%) dead fish were mutant fish, no significant difference in mutation rate between dead and alive fish was found in this family (P=0.816). Body weight was not significantly different between mutant fish and non-mutant fish (P=0.857) for these dead fish (Table 4).

Table 3. Comparisons of the embryo hatch rate and the 6-months-old fingerlings survival rate between P_1 transcription activator-like effector nuclease (TALEN) plasmid treatment fish and the full-sibling controls. Comparisons of the survival rate of 3-year-old and 4-year-old P_1 mutant channel catfish (*Ictalurus punctatus*) and their full-sibling treatment non-mutant fish cultured in recirculating systems and earthen ponds.

Treatment	P_1 embryo and 6-month-old fingerlings of treatment group and control group				Genotype	3-year-old mutant and non-mutant P_1 fish in the recirculating system in 2016		4-year-old mutant and non-mutant P_1 fish in 2017			
								Recirculating system		Pond	
	N eggs	N hatched	Hatch rate (%)	Survival rate (%)		N fish	Survival rate (%)	N fish	Survival rate (%)	N fish	Survival rate (%)
Electroporated with TALEN plasmids	200	76	38.0	67.1	Mutants	42	50.0	12	41.2	32	46.9
Electroporated without TALEN plasmids (control)	200	84	42.0	75.0	Non-mutants	18	33.3	8	37.5	6	33.3

Notes:
Hatch rate and survival rate were not significantly different between the treatment group and control group for P_1 embryo and 6-months fingerlings (Fisher's exact test, P=0.475, P=0.223).
Survival rates were not significantly different between mutants and non-mutants for 3-year-old P_1 and 4-year-old P_1 in the recirculating system and pond, respectively (Fisher's exact test, P=0.270, P=1.000, P=0.672).

Table 4. Survival rate, mutation rate and mean body weight of the four families (2016 GnRH-1, GnRH-2, GnRH-3 and GnRH-4) of 1.5-year-old F_1 offspring spawned in 2016 without hormone therapy, and the three families (2017 GnRH-1, GnRH-2 and GnRH-3) of 1-year-old F_1 offspring spawned in 2017 with hormone therapy of channel catfish (*Ictalurus punctatus*). Mutant males and females were paired with wild type fish to generate F_1 offspring in 2016. Mutant males were paired with mutant females to generate F_1 offspring in 2017. The fish in the 2017 GnRH-1 family died from columnaris (*Flavobacterium columnare*). The survival rate for this family was calculated after the outbreak of columnaris. Mean body weight data were presented as the mean ± standard error (*SEM*).

Different Families of F_1 offspring	Survival rate			Mutation rate			Mean body weight (g) ± *SEM* of mutant fish	Mean body weight (g) ± *SEM* of non-mutant fish
	N fish	N fish survived	Survival rate (%)	N fish sampled	N mutant fish	Mutation rate (%)		
2016 GnRH-1	30	14	46.7	30	18	60.0	164.5±5.4	166.6±7.8
2016 GnRH-2	30	12	40.0	30	19	63.3	154.3±6.3	161.9±12.4
2016 GnRH-3	30	22	73.3	30	23	76.7	70.7±3.7	68.3±6.3
2016 GnRH-4	30	16	53.3	30	20	66.7	154.9±8.5	170.5±11.8
2017 GnRH-1	300	224	74.7	100	53	53.0	6.1±0.2	5.9±0.2
2017 GnRH-1 fish died from columnaris	/	/	/	56	36	64.3	13.13±0.5	13.30±0.8
2017 GnRH-2	200	185	92.5	30	14	46.7	10.0±0.7	9.4±0.6
2017 GnRH-3	300	274	91.3	100	51	51.0	6.8±0.2	6.7±0.2

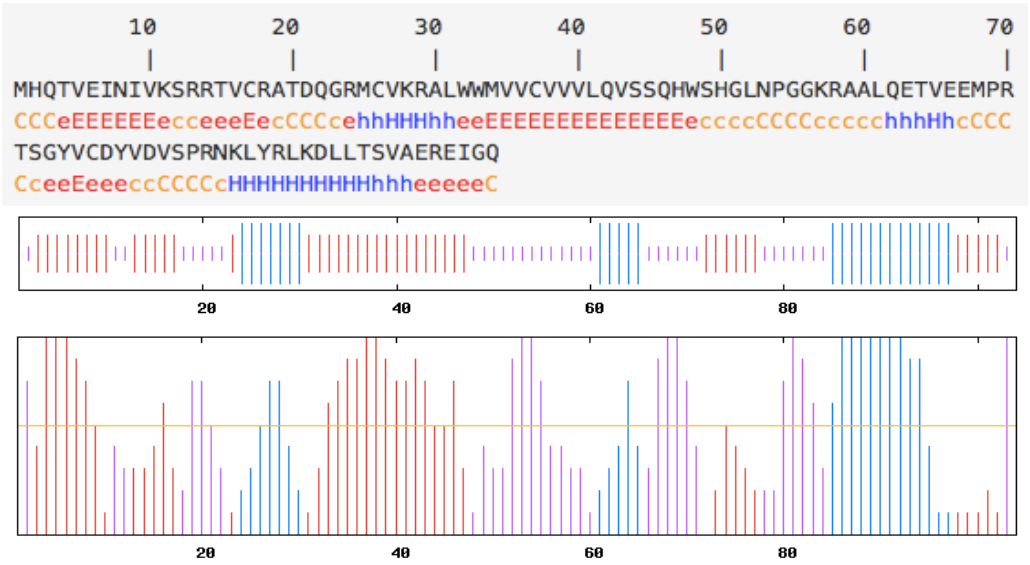
Notes:
Survival rate was significantly different between F_1 families with hormone therapy in 2016, and without hormone therapy in 2017 (Student's *t* test, P=0.014).
Mutation rate was significantly different between F_1 families with hormone therapy in 2016, and without hormone therapy in 2017 (Student's *t* test, P=0.013).
Body weight was not significantly different between mutant fish and non-mutant fish in each F_1 families, including 2016 GnRH-1, 2016 GnRH-2, 2016 GnRH-3, 2016 GnRH-4, 2017 GnRH-1, 2017 GnRH-2 and 2017 GnRH-3 (Student's *t* test, P=0.826, P=0.598, P=0.749, P=0.296, P=0.321, P=0.563, P=0.927).
Body weight was not significantly different between the dead diseased mutant fish and non-mutant fish in 2017 GnRH-1 F_1 family (Student's *t* test, P=0.857).
Mutation rate was not significantly different between 2017 GnRH-1 family and the fish that died from columnaris in this family (Fisher's exact test, P=0.816). The mutation rate of the non-diseased fish was calculated before the outbreak of columnnaris.

3.5 Sequence Structure Analysis

There are total 104 amino acids of cfGnRH protein, and 46 amino acids of GAP. The target alignment shows insignificant similarity score for both cfGnRH and GAP. The predicted secondary protein structure of cfGnRH protein and GAP were predicted using PBIL. The secondary structure of cfGnRH protein was categorized in alpha helix (24.04%), extended strand (40.38%) and random coil (35.58%) (Fig. 5A). As shown in Fig. 5B, sequence and secondary structure analyses of cfGnRH showed that it has 20 β-turns, 6 γ-turns and 3 α-helices.

Meanwhile, the tertiary structure of cfGnRH was identical to 3u84A with C-score of -3.58, and the image was colored by rainbow from N to C terminus (Fig. 5C).

A



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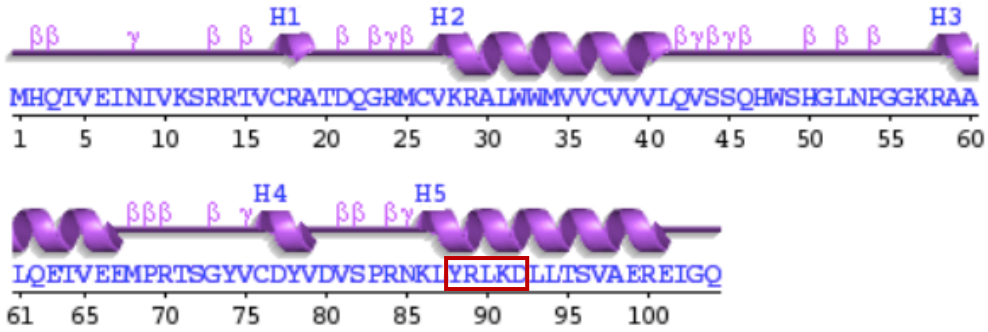

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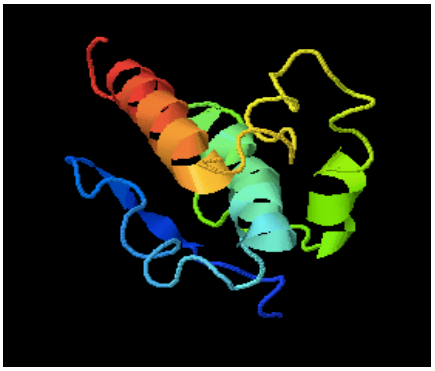
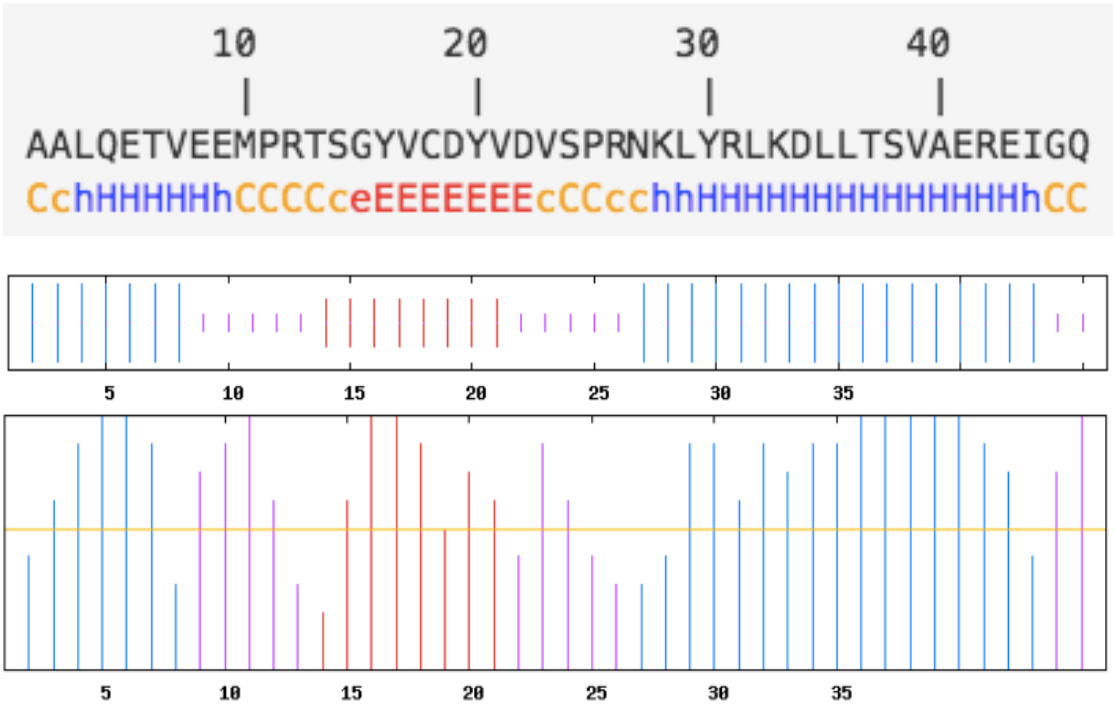


Figure 5. Prediction and analysis of catfish type gonadotropin-releasing hormone (cfGnRH) protein structure. **(A)** Prediction and analysis of secondary structure of cfGnRH protein using Pole Bioinformatique Lyonnais (PBIL) server, which was categorized in alpha helix (24.04%), extended strand (40.38%) and random coil (35.58%) Hh: Alpha helix; Ee: Extended strand; Cc: Random coil. **(B)** Secondary structure of cfGnRH was predicted using PDBsum Generate, which has 20 β-turns, 6 γ-turns and 3 α-helices. Transcription activator-like effector nuclease (TALEN) targets the 5 amino acids of "YRLKD". Rex box represents the targeted amino acids. **(C)** Three-dimensional structure of cfGnrH was predicted using I-TASSER server, which is identical to 3u84A with C-score of -3.58, and the image was colored by rainbow from N to C terminus.

The TALENs targets 5 amino acids, "YRLKD", on the GAP region. For GAP, the secondary structure of GAP was categorized in alpha helix (52.17%), extended strand (17.39%) and random coil (30.43%) (Fig. 6A). As shown in Fig. 6B, sequence and secondary structure analyses of GAP showed that it has 11 β-turns and 1 γ-turn. Meanwhile, the tertiary structure of cfGnRH was identical to 4d7sA with C-score of -2.36, and the image was colored by rainbow from N to C terminus (Fig. 6C).

A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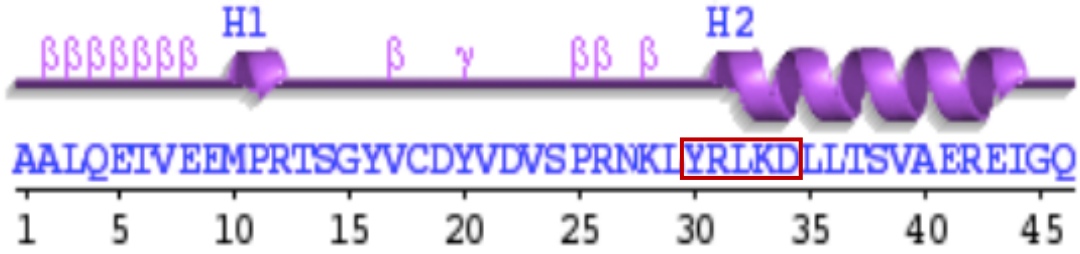
B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H2
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1
5
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25
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35
40
45

C

Figure 6. Prediction and analysis of gonadotropin-releasing hormone-associated peptide (GAP) structure. **(A)** Prediction and analysis of secondary structure of GAP protein using Pole Bioinformatique Lyonnais (PBIL) server, which was categorized in alpha helix (52.17%), extended strand (17.39%) and random coil (30.43%) Hh: Alpha helix; Ee: Extended strand; Cc: Random coil. **(B)** Secondary structure of GAP was predicted using PDBsum Generate, which has 11 β-turns and 1 γ-turn. Transcription activator-like effector nuclease (TALEN) targets the 5 amino acids of "YRLKD". Rex box represents the targeted amino acids. **(C)** Three-dimensional structure of cfGnrH was predicted using I-TASSER server, which is identical to 4d7sA with C-score of -2.36, and the image was colored by rainbow from N to C terminus.

4 Discussion

Heritable mutations were detected at the targeted site of the cfGnRH gene. Different types of mutations were identified, including base deletion, substitution, and insertion with the total number of base changes ranging from 1-5. Theoretically, base deletion and insertion could shift the codon sequence, altering the open reading frame, changing the downstream amino acid sequence, and increasing the probability of premature stop codons. The base substitution could introduce a stop codon or change a codon to one that encodes a different amino acid and cause the change in the protein produced. In the P_1 generation, the mutation rate of TALEN-exposed 6-month-old fingerlings was 52.9%. For the mutant fish, the spawning rate, reproductive behavior and the hatch rate of their offspring were suppressed. Spawning rate of mutant fish was lower than wild types, especially for ovulation rate when they were mated without hormone-induced spawning. Average egg hatch rate was 2.0% except for one cfGnRH mutated female that had a 66.0% hatch rate. Hormone therapy restored the fertility of mutant P_1 fish in 2017. No pleiotropic effects on body weight and survival rate were detected in mutant fish. This is the first sterilization achieved using TALENs technology targeting GAP region in an aquaculture species.

GAP is the C-terminal portion of the GnRH preprohormone. Sirkin et al. (2017) showed that type 1 GAP peptide presented a typical helix-loop-helix (HLH) structure in all the vertebrate species analyzed. In this study, the catfish type GnRH also presented a HLH structure. The conservation of the HLH motif, known to confer biological activity to various proteins, suggested that GAP peptides may exert some hypophysiotropic biological function in channel catfish.

TALENs have been applied as an effective method of mutagenesis in a variety of species using microinjection. TALEN application resulted in mutation rates ranging from 13% to 67% in mice (Qin et al. 2013). Recent large-scale studies have reported TALENs mutation efficiencies between 3% and 95% (Kim et al. 2013, Reyon et al, 2012). The mutation rate via electroporation for the cfGnRH gene, 52.9%, was relatively high compared to other studies through electroporation. Two to three hundred one-cell stage embryos can be microinjected in one batch of eggs (Elaswad et al. 2018), while at least four hundred one-cell stage embryos can be electroporated in one batch of eggs . Electroporation was an effective and simple technique to utilize TALEN technology to induce mutations in channel catfish, and this can also be easily adapted for other fish species. There was no evidence for adverse off-target effects. These results appear promising for application in large-scale, commercial aquaculture.

In zebrafish, the GnRH3 gene knocked out fish are still fertile, displaying normal gametogenesis and reproductive performance in males and females (Spicer et al. 2016). The loss of both GnRH2 and GnRH3 isoforms resulted in no major impact on reproduction, indicating that a compensatory response, outside of the GnRH system was evoked (Marvel et al. 2018). In medaka (*Oryzias latipes*), none of the female GnRH1 gene knocked out fish could ovulate, while all male GnRH1 gene knocked out fish were fertile. Our results in channel catfish are different

from both zebrafish and medaka. In this study, the spawning rate of mutants was lower than wild types, especially for ovulation rate when they were mated without hormone induced spawning. Average egg hatch rate was 2.0% except for one cfGnRH mutated female that had a 66.0% hatch rate.

In 2016, all the wild type females were crossed with mutant males, but abnormal courtship was observed through the bottom of the transparent aquaria. These results indicated two forms of GnRH may interact with each other to control the reproductive behaviors of channel catfish. Additionally, some wild type females laid immature eggs without generating an egg mass. The lack of normal reproductive behavior by mutant males could also have contributed to wild type females responding inappropriately. LHRHa hormone therapy resulted in good spawning and hatch rates for mutants. Three of five mutant males and three of six mutant females exhibited normal reproductive behavior. P_1 spawning rate was increased, and hatch rate was increased significantly compared with the results in 2016 before hormone therapy, excluding the 3 pairs in 2016 that did not spawn with hormone therapy likely due to the lateness of the spawning season and perhaps over ripeness. The lower survival rate in 2016 F_1 families may be because of the lingering maternal effect due to poor egg quality, or the environmental effect during the year or the combination of these two possible reasons. More research is needed to find the answer.

Cell toxicity can be an issue when the genome is edited. Cell death and apoptosis are most likely affiliated with off-target effects. Generally, TALENs are designed to target genes with high specificity and low cytotoxicity (Dreyer et al. 2015, Mussolini et al. 2014, Veres et al. 2014). For the P_1 generation, both the embryo hatch rate and fry survival rate of the TALEN treatment group was not different from the control group, which suggests that the TALEN

plasmids and the nucleases had high specificity and only targeted the cfGnRH gene with little, if any, off-target mutations. After three to four years, no differences in survival were found between P_1 mutant fish and their full-sibling controls cultured in recirculating systems and ponds further confirming this conclusion.

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Ⅱ Gene Editing of the Follicle Stimulating Hormone Gene and Hormone Therapy to Control the Reproduction in Channel Catfish, *Ictalurus punctatus*

Abstract

Follicle stimulating hormone (FSH) plays an important role in sexual maturation in catfish. Knocking out FSH gene in the fish zygote should suppress the reproduction of channel catfish (*Ictalurus punctatus*). In this study, transcription activator-like effector nucleases (TALENs) plasmids targeting the FSH gene were delivered into fertilized eggs with the double electroporation. Targeted cleavage efficiency was 63.2% in P_1 fish. Ten of 15 (66.7%) control pairs spawned, and their eggs had a 32.3-74.3% average hatch rate in 2016 and 2017. P_1 mutants had spawning rates of 33.3-40.0% when there was no hormone therapy with average egg hatch rates of 0.75%. After establishing low fertility in 2016, human chorionic gonadotropin (HCG) hormone therapy resulted in spawning rates of 80% for female mutants and 88.9% for male mutants, and mean hatch rate of 35.0% for F_1 embryo, which were not significantly different from controls ($P>0.05$). Polymerase chain reaction (PCR) inspection showed no potential TALENs plasmid integration into P_1 channel catfish genome. No obvious off-target effects on body weight, survival rate and hatch rate were observed after the knockout of reproductive genes in the P_1 and F_1 mutant fish, also a higher survival rate was found in mutants of 4-year-old P_1 fish ($P<0.05$). F_1 families had mean inheritance rate of 50.3%. The results placed us one step closer to allowing application of certain aquaculture and fisheries management genetics

techniques while essentially eliminating the potential environment risk of transgenic, hybrid and exotic fish as well as domestic fish.

1 Introduction

Genetically engineered catfish have great potential for higher yield, better disease resistance, greater survival ability in extreme environments and the secretion of specific proteins (Dunham et al. 1992, 1999, 2002). However, concerns have been expressed regarding the potential ecological and genetic effects of these fish (Hedtrick 2001). An effective fish sterilization technology could prevent or minimize environmental risk of transgenic fish.

Previous studies have shown that the maturation of gametes in the male and female fish is influenced by gonadotropins, including FSH and LH, which regulate in vitro synthesis and the secretion of steroid hormones to induce oocyte maturation and spermatogenesis (Patino et al. 1990, Prat et al. 1996, Swanson et al. 1991, Swanson et al. 2003, Kwok et al. 2005). FSH is one of the gonadotropins that belongs to the glycoprotein hormone family (Hsu et al. 2002). Glycoproteins are functional heterodimers, consisting of one α subunit and one β subunit. Within a given fish species all α subunits are identical while the different β subunit confers the physiological specificity of the hormone (Liu et al. 2001). The maximum levels of the synthesis and secretion of FSH correspond to the stage of vitellogenesis in the annual reproductive cycle in salmon, whereas LH peak production occurs during oocyte maturation and ovulation (Dolomatov et al. 2012). FSH levels are elevated during spermatogenesis while LH levels peak in the spawning season in salmonid fish (Schulz and Henk 1999). Simultaneous presence of ovarian LH and FSH levels are needed during the reproductive cycle due to the presence of oocytes that are at different stages of maturation (Ko et al. 2007). The functioning reproductive system of fish

is not only seen in the rhythms of the neuroendocrine activity level, but also in the dynamics of gonadotropin receptor expression in the gonads (Kwok et al. 2005). In addition to gonadotropins, growth hormone and insulin like growth factor (IGF1) also influence the process of maturation of the ovaries, which contributes to a more rapid rate of maturation of eggs, and increase the biosynthesis of the sex steroids that are responsible for the maturation (Campbell et al. 2006). Hypothetically, the mutations of FSH gene could affect the normal function of the gene and lead to sterilization of fish.

The objectives of this study were to deliver TALENs plasmids targeting the FSH gene into eggs and fertilized eggs with double electroporation technique. The gene edited, sterile channel catfish (*Ictalurus punctatus*) was developed via TALEN mediated mutagenesis on the genomic level. Fish mating experiments were utilized to evaluate the reproductive capability of mutant catfish. Mutation analysis were employed on F_1 fish to verify the heredity of mutations. Reversal of sterility of gene edited fish is achievable through hormone therapy. No significant pleiotropic effects were found in the F_1 generation. The successful development and demonstration of this sterilization technology could be used for bioconfinement of catfish and other species of gene engineered fish, as well as domestic and invasive fish.

2 Materials and Methods

2.1 Construction of Plasmid

Transcription activator-like effector nucleases (TALENs) are proteins selected from the bacteria *Xanthamonas*. *Xanthomonas* transcription activator-like effector nucleases (XTNTMs) plasmids were designed and assembled by Transposagen Company (Lexington, KY). Each XTN was cloned into the SQT281 vector in the TAL repeat region and driven by the cytomegalovirus

(CMV) promoter, T7 promoter, then followed by FokI nuclease domain (Fig. 7). The TALENs target sites were designed to be located at the β subunit of FSH gene (GenBank Accession No. NM_001200079.1), which confers the physiological specificity of the hormone. Each TALEN comprised an 18 bp (5'- TACCAACATCTCCATCAC- 3') left DNA binding site, or an 18 bp (5'- TGTGGCAGCTGCATCA- 3') right DNA binding site.

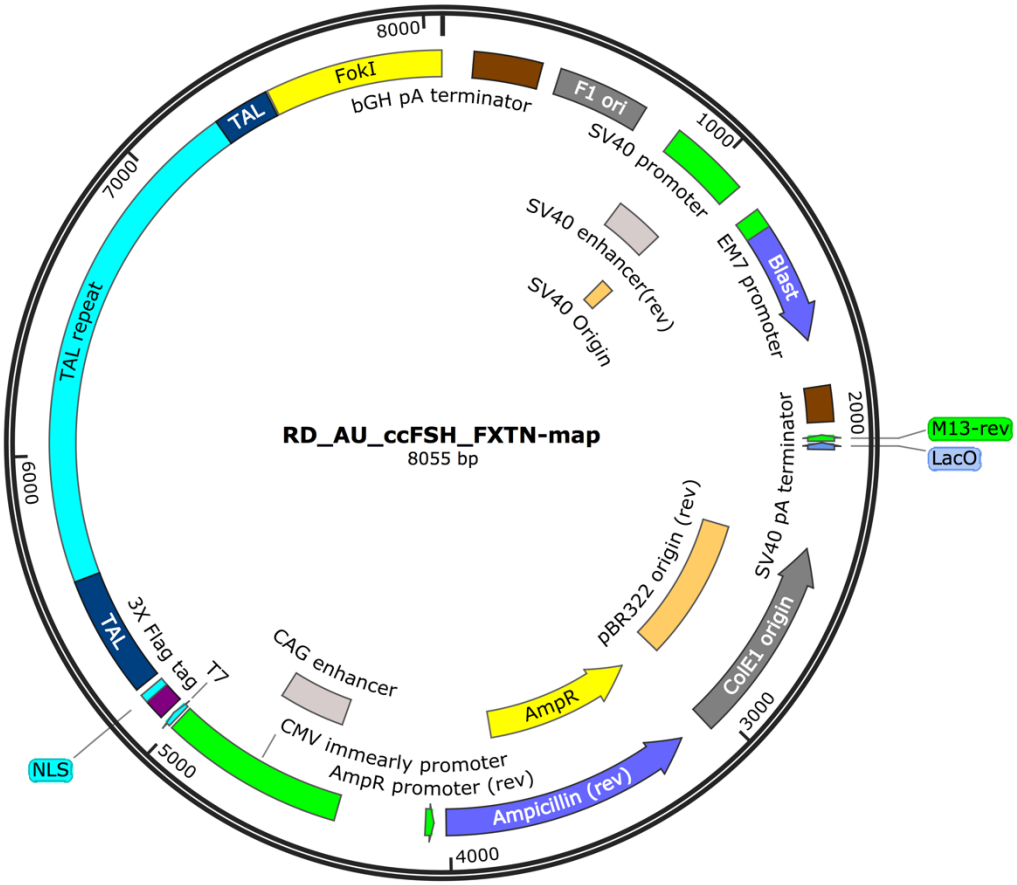


Figure. 7 Schematic representation of transcription activator-like effector nucleases (TALENs) plasmid structure targeting follicle stimulating hormone (FSH) gene of channel catfish (*Ictalurus punctatus*). *CMV prmt* cytomegalovirus promoter, *NLS* nuclear localization signal, *BGH pA* bovine growth hormone and polyadenylation signal, *AmpR* ampicillin resistance gene.

2.2 TALEN Plasmid Replication, Extraction and Dilution

The TALEN plasmids were transformed into One Shot® TOP10F′ Competent Cells (Invitrogen, Grand Island, NY), following the transformation procedures from the manufacturer. One hundred μL of each transformation mix vial was spread on the LB agar plate with 100 μg ml^{-1} ampicillin separately. A single colony was picked from each plate and then cultured in 400 mL LB broth with 100 μg ml^{-1} ampicillin separately. Then the TALEN plasmids were extracted with the IsoPure Plasmid Maxi II Prep Kit (Denville, Holliston, MA). DNA agarose gel electrophoresis and spectrophotometry were then used to test the quantity and quality of the TALEN plasmids. Equal amounts of both left and right TALEN plasmids were mixed together. They were diluted with 2 mL saline (0.9% NaCl) to obtain a 25 μg ml^{-1} mix solution and diluted with 5 mL TE buffer (5mM Tris-HCl, 0.5M EDTA, pH=8.0) to collect a mix solution with the same concentration, respectively.

2.3 Experimental Brood Stock and Gametes

Sexually mature females of Kansas Random strain of channel catfish (*Ictalurus punctatus*) were selected and implanted with luteinizing hormone releasing hormone analog (LHRHa) (Reproboost® Implants, Center of Marine Biotechnology, Columbus Center, Maryland, USA) with a dose of 90 μg kg^{-1}. All showed outstanding secondary sexual characteristics. Eggs of two anesthetized ovulating females were hand stripped into dried stainless steel pie pans greased with vegetable shortening. Two-hundred eggs were collected for each experimental group and control group. One Kansas Random strain male and one Auburn-Rio Grande strain male (Dunham and Smitherman, 1983) were euthanized. Mixed testes were placed into a mesh strainer and macerated manually into saline (0.9% NaCl) to collect sperm.

2.4 Fertilization, Electroporation and Incubation

The first electroporation was conducted on the collected sperm homogenate with the TALEN saline solution using a Baekon 2000 macromolecule transfer system (Baekon, Inc., Saratoga, CA) with parameters of 6kV, 27 pulses, 0.8s burst, 4 cycles and 160μs (Su et al. 2013). Then two hundred prepared eggs were artificially fertilized with the electroporated sperm. After one hour, the embryos were gathered and immersed with the TALEN TE buffer solution for ten minutes. Then the second electroporation was conducted with the same procedure. The control group was generated following the same protocols, but with the absence of TALEN plasmids. Embryos were then moved into 10 L tubs with Holtfreter's solution containing 10 ppm doxycycline and incubated statically until hatch. Dead embryos were removed daily before Holtfreter's solution was changed.

2.5 Sample Collection and Mutation Analysis for P_1 generation

Embryos were cultured for 6-7 days until hatch in tubs at 27°C and then transferred into a recirculating system. After 6 months, the genomic DNA was extracted from the pelvic fin and barbel samples. Samples were digested with cell lysis buffer and proteinase K following the protocols by Cheng et al. (2014). DNA agarose gel electrophoresis and spectrophotometry were then used to test the quantity and quality of the genomic DNA. Then Roche Expand High FidelityPlus PCR System (Roche, Indianapolis, IN) was used to amplify the channel catfish FSH gene on these DNA samples. The following primers were utilized: forward sequence 5'-CACAACTCCAGCTGTGACAA -3'; reverse sequence 5'- CAGAATTCCGTGGCCATTTA -3' (Table 1), and the amplification procedure was: initial denaturation for 2 min at 94°C,

followed by 29 cycles of 94°C for 30s, 60°C for 30s and 72°C for 40s, and the final elongation for 10min at 72°C.

Gene mutations were detected using Surveyor® Mutation Detection Kit for Standard Gel Electrophoresis (Integrated DNA Technologies, Coralville, IA) (Qin et al. 2015). The key component of the kit is Surveyor Nuclease, a member of the CEL family of mismatch-specific nucleases derived from celery, which can be used to perform a highly specific cleavage on the 3' side near the mutation site of the two strands of the heteroduplex DNA. The recognized mutant forms include single base or multiple base insertions, deletions or mismatches (Qiu et al. 2004). Surveyor Nuclease has been a powerful and repeatable tool for mutation testing. It has been used to detect mutations and polymorphism in human, mammal, bacteria, and plant genomes (Pimkin et al. 2004; Tsuji and Niida, 2008). The processes were as follows: first, the PCR products was hybridized using a PCR machine to form a heteroduplex using a thermal cycler as 95°C 10min, 85°C 1min Ramp 2°C/s, 75°C 1min Ramp 0.3°C/s, 65°C 1min Ramp 0.3°C/s, 55°C 1min Ramp 0.3°C/s, 45°C 1min Ramp 0.3°C/s, 35°C 1min Ramp 0.3°C/s and 25°C1 min Ramp 0.3°C/s; then a nuclease reaction system, which included DNA hybridization system, 150mM $MgCl_2$, 5×PCR buffer, Surveyor enhancers S and Surveyor nucleases were established and incubated in a PCR machine under 42°C for 1 hour; finally, the 2% UltraPure Agrose-1000 (Invitrogen, Grand Island, NY, USA) was used to perform electrophoresis to test the mutation.

2.6 TA Clone and Sequencing

To confirm the presence of the mutations, Roche high fidelity PCR amplification targeted FSH gene was performed with the sample DNA. Then TOPO® TA Cloning® kit was used to set up a 6μL TOPO cloning system, which included 4μL amplified DNA product, 1μL salt solution

and 1μL TOPO vector. The system was mixed and incubated under room temperature (22-23°C) for 5min, followed by competent cell transfection. First, 2μL of the cloning system was added into one vial of One Shot®TOP10F'competent cells and put on the ice for 30min. Then 42°C heat shock was implemented using PCR machine for 30s. After that, the tube was placed on ice immediately. The next, 250μL LB (Tryptone 10g/L, Yeast extract 5g/L, NaCl 10g/L, Agarose 10g) broth was mixed together in a 1.5ml tube and placed in the 37°C shaker and shaken at 200rpm for 1 hour. Next, 50μL, 80μL, and 100μL mixtures were spread on the LB (Tryptone 10g/L, Yeast extract 5g/L, NaC l10g/L, Agarose 10g) medium and placed in the 37°C incubator overnight. Ten monoclonal colonies were picked from each plate, mixed with 400ml LB (Tryptone 10g/L, Yeast extract5g/L, NaCl 10g/L, Agarose10g) broth and placed in the 37°C incubator overnight. The amplified bacterial solution was dispensed into a 96-wells plate and sent to the Eurofins Genomics Company (Louisville, KY, USA) for sequencing. Finally, sequencing results were analyzed using T-Coffee tool (Notredame et al. 2000).

2.7 Plasmid Integration Inspection

PCR was conducted to determine if plasmids were integrated into the genome or persisted in the cytoplasm. Two pairs of primers targeting FSH gene were designed to detect the plasmid DNA in mutant fish. The amplification regions were CMV promoter region and TAL repeats region respectively. They were amplified using the primers as shown in Table 5. The amplification procedure was as follows: initial denaturation for 2 min at 94°C, followed by 29 cycles of 94°C for 30s, 60°C for 30s and 72°C for 40s, and the final elongation for 10min at 72°C. The results were generated using electrophoresis.

Table 5. Primer sequences used for the amplification of follicle stimulating hormone (FSH), cytomegalovirus (CMV) promoter and transcription activator-like (TAL) repeats regions in channel catfish (*Ictalurus punctatus*).

Targeting Site		Sequence
FSH	Forward	5'-CACAACTCCAGCTGTGACAA-3'
	Reverse	5'-CAGAATTCCGTGGCCATTTA-3'
CMV promoter	Forward	5'-GCAAATAATAACGGTGGCAA-3'
	Reverse	5'-GTTTCCCTCCGTCATGCG-3'
TAL Repeats	Forward	5'-GCGAATAACAATGGAGGGA-3'
	Reverse	5'-GCCACCGTTATTATTTGCAA-3'

2.8 Reproductive Evaluation

The fingerlings were transferred to 0.04- hectare pond or a recirculating system when they reached 15-20cm for further growth and maturation. Mating experiments began on June 20, 2016. There were 5 mutant (M) males and 3 mutant females selected to mate with wild type (Wt) females and males (Wt♀×M♂, M♀×Wt♂). There were five pairs of control of Kansas Random strain of channel catfish selected for natural spawning without hormone injection. These fish were paired in 48cm×36cm×21cm aquaria with constant water flow and compressed air for aeration. All fish showed outstanding secondary sexual characteristics. The sexually mature males had well-developed head muscles and dark mottleing in the lower jaw and abdomen. The sexually mature females exhibited fuller, rounder abdomen and reddening genital area compared to immature females.

For testing the fertility of mutant males, only the paired wild type females were implanted with 75μg/kg LHRHa for induced spawning. Then the wild type females that did not spawn within 8 days were implanted again with 90μg/kg LHRHa. If the females did not spawn after two implantings, they were replaced by other wild type females and the hormone implantation for laying eggs. For testing the fertility of mutant females, the pairs were given 17 days to spawn naturally with the wild type males and without hormone injection.

2.9 Hormone Therapy

In 2016, 2 mutant males and 2 mutant females mated with each other and implanted with 50μg/kg LHRHa and 300IU/kg pregnant mare gonadotropin (M♀×M♂). After establishing infertility in 2016, hormone therapy was conducted in 2017. There were 9 mutant males and 10 mutant females with outstanding secondary sexual characteristics selected to mate with each other (M♀×M♂). Both females and males were injected with 1200IU human chorionic gonadotropin (HCG). If females and males did not spawn within 5 days, they were injected again with 400IU HCG. Five pairs of control of Kansas Random strain of channel catfish were selected for natural spawning without hormone injection.

2.10 F_1 Fish Culture

When egg masses were obtained, they were placed in baskets in hatching troughs with constant water flow and aeration. Calcium chloride solution was continually dripped into the trough to ensure 40-50ppm hardness (Steeby, 2005). Eggs were gently agitated with a paddlewheel beginning 2 hours after spawn collection. The egg masses were prophylactically treated with 100ppm formalin or 32ppm copper sulfate every 8 hours to avoid fungus (Small et

al. 2006). The treatments were terminated 12 hours before hatch.

Catfish embryos began hatching in 7 days with a water temperature between 26-28℃. They consumed their yolk sac and began swim up stage 3 days post hatching. They were then fed artemia (Brine Shrimp Eggs, Carolina Biological) three times a day and stocked into a recirculating system with densities of 1000 fish per 90L tank and 100 fish per 60L aquaria of each family. After one month fry were fed Purina® AquaMax® Fry Powder (Purina, St. Louis, MO) three times a day. As fry grew they were then fed Purina® AquaMax® Fry Starter 100. After further growth fry were moved to 90L tank at a stocking density of 500 fish per tank and fed Purina® AquaMax® Fry Starter 200 and 300 three times a day.

2.11 Sample Collection and Mutation Analysis for F_1 generation

When 2016 spawned F_1 fish were 1.5-year-old, ventral fin samples were collected from 30 fish in each family. When 2017 spawned F_1 fish were 1-year-old, ventral fin samples were collected from 20-30 fish in each family. Surveyor mutation analysis was conducted. The procedures were the same as described above.

2.12 Pleiotropic Effects

For P_1 fish, survival rate for embryos, 6-month-old fingerlings and 4-year-old adult fish was measured. For F_1 fish, survival rate and body weight for F_1 1-year-old and 1.5-year-old fish were measured. Thirty dead fish from a low dissolved oxygen accident in one tank were collected. The reproductive behavior was observed through the bottom of overhead aquariums.

2.13 Statistical Analysis

Statistical analysis of hatch rate, survival rate and body weight were performed with R studio software (RStudio Inc, Boston, MA). Mutation rate, survival rate, and hatch rate were compared utilizing Fisher's exact test in case of small sample size. Student's *t*-test was performed to compare hatch rate and spawning rate between treatments and controls for P_1 fish. Student's *t*-test was also applied to compare survival rate and mutation rate between F_1 families before and after hormone therapy, and compare body weight in each F_1 families. Shapiro-Wilk's test was used to test the normality of the data. Comparisons of statistical significance were set at $P<0.05$, and all data were presented as the mean ± standard error (*SEM*).

2.14 Sequence Structure Analysis

FSH mRNA sequences were retrieved from the nucleotide NCBI database (https://www.ncbi.nlm.nih.gov/). Amino acid sequences of FSH protein were translated using ExPASy software (https://web.expasy.org/translate/). Sequences of FSH protein was used as queries to BLAST against protein data bank (PDB) database with a cutoff E-value of $1e\text{-}^{10}$. If it shows significant similarity with query sequence, then the secondary structures of FSH protein was predicted using PDBsum software (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl?pdbcode=index.html). If it doesn't show significant similarity with query sequence, then the secondary structures of FSH gene was predicted using Pole Bioinformatique Lyonnais (PBIL) server (https://prabi.ibcp.fr/htm/site/web/home). The three-dimensional protein structure was predicted using the I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). Only models with the C-score between 2 and -4 were considered. C-score is a confidence score for estimating the quality of predicted models. It is calculated based on the significance of threading template alignments and the

convergence parameters of the structure assembly simulations. C-score is typically in the range of [-5, 2], where a C-score of higher value signifies a model with a high confidence and vice-versa. The visualization of predicted three-dimensional structures was performed using the Jmol software. The predicted model was downloaded from I-TASSER as a PDB file. The predicted secondary protein structure based on the predicted 3D model was displayed using the PDBsum server and compared with the result from PBIL.

3 Results

3.1 Analysis of Mutation Efficiency and Plasmid Integration of P_1 Generation

The target cleavage efficiency was 54.4% in 31 of 57 6-month-old P_1 fingerlings. The surveyor mutation assay results of pelvic fin and barbel tissues showed five bands on gel. The 350bp band indicated the FSH gene was cut at the expected site (Fig. 8).

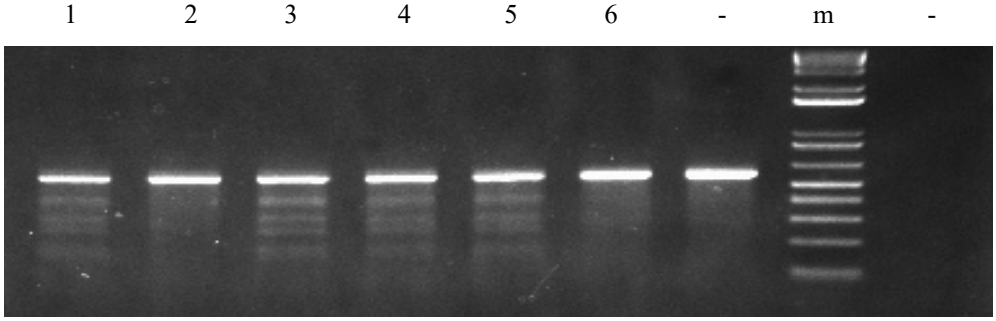


Figure. 8 Identification of edited follicle stimulating hormone (FSH) gene in P_1 channel catfish (*Ictalurus punctatus*) using Surveyor mutation detection assay. The left "-" indicates the negative control with wild type template. The right "-" indicates the negative control without DNA template; "m" indicates 1kb DNA ladder; 1, 3, 4, 5 are channel catfish with mutation; Lane 2 and 6 are channel catfish without mutation.

Sequence and alignment analyses confirmed that channel catfish (*Ictalurus punctatus*) were successfully mutated for the FSH gene. All the mutations occurred within the target TALEN cutting site. Different types of mutation were found, including base deletions, substitutions and insertions. These mutations were in the coding region of the β subunit of FSH gene, which confers the physiological specificity of the hormone. These mutations could alter the product of FSH gene, or prevent the gene from functioning properly or completely. (Fig. 9 and Fig. 10).

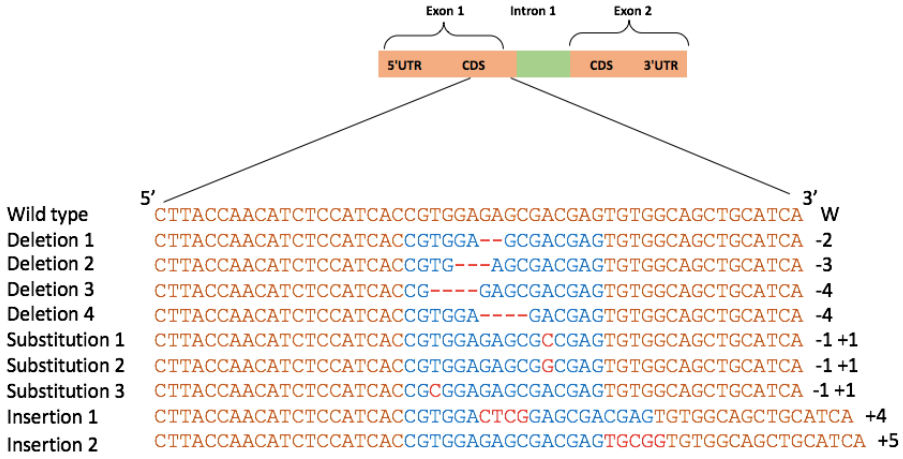


Figure. 9 Sequences of follicle stimulating hormone (FSH) gene in channel catfish (*Ictalurus punctatus*). The wild-type channel catfish FSH gene sequence is showed on the top. Sequences in orange are the target binding sites of the transcription activator-like effector nucleases (TALENs). Sequences in blue are the expected cleavage sites of the nucleases. Red dashes and letters indicate the deletion/insertion/substitution of nucleotides. Numbers at the end of the sequences show the number of nucleotides deleted (-) or inserted (+) in the edited FSH gene.

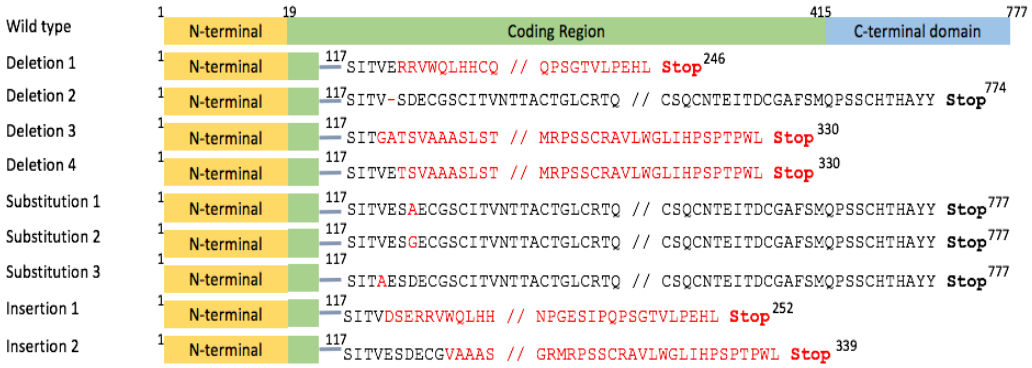


Figure. 10 Predicted amino acid sequences of follicle stimulating hormone (FSH) gene produced from the mutant channel catfish (*Ictalurus punctatus*). Amino acid sequences following incomplete domain were due to fame-shift reading, resulting in a premature stop (red color) codon. Single amino acid substitutions or deletions (red color) were due to single nucleotide substitution mutation or three nucleotides deletion.

Based on the plasmid integration inspection, neither CMV promoter region nor TAL repeats region was amplified, which indicated that no plasmid DNA was detected with PCR for all TALEN-FSH mutant fish. The results indicated that none of the fingerlings carried the exogenous DNA (Fig. 11).

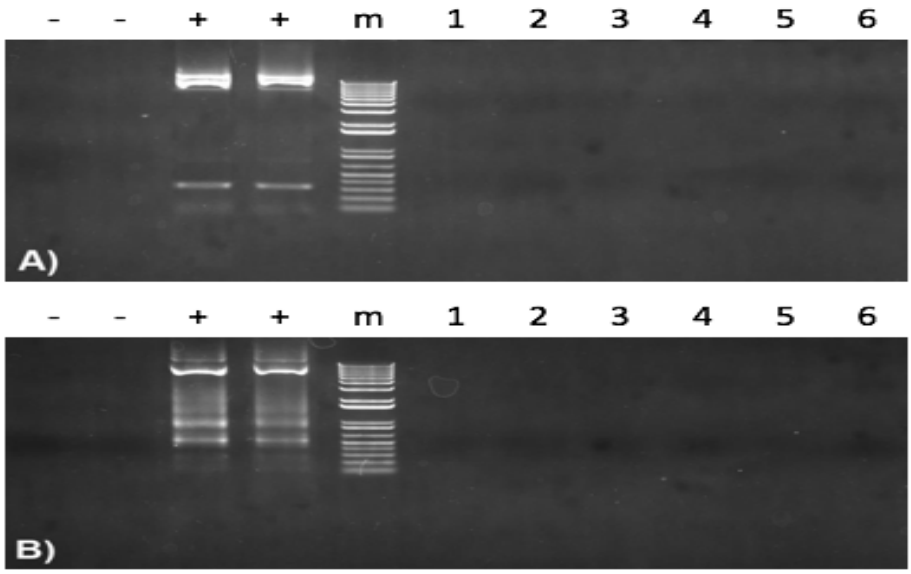


Figure. 11 Polymerase chain reaction (PCR) inspection of potential transcription activator-like effector nucleases (TALENs) plasmid integration into channel catfish (*Ictalurus punctatus*) genome. The left two lanes of "-" indicates the negative controls without template, the right two lanes of "-" indicates the negative controls with wild-type channel catfish as template; "+" indicates the positive controls with TALEN left and right plasmids as template, respectively; "m" indicates 1 kb DNA ladders. Numbers represent channel catfish individuals carrying mutated FSH gene; the same number indicates the same individual. A) & B) represent the PCR detection with different specific primers designed to amplify cytomegalovirus (CMV) promoter region and transcription activator-like (TAL) repeats region, respectively.

3.2 Spawning Experiments and Hormone Therapy

Three of 5 (60.0%) control pairs spawned with a 74.3% average hatch rate in 2016. Five of 7 (71.4%) control pairs spawned with a 32.3% average hatch rate without LHRHa hormone injection, while 2 of 3 (66.7%) control pairs spawned with an 56.5% average hatch rate with LHRHa hormone injection in 2017. P_1 mutants had spawning rates of 33.3% for females and 40% for males when there was no hormone therapy with egg hatch rates of 0.45-1.0%. The hatch rate between the mutant group and control group was significantly different (P=0.001).

In 2017, 1200 IU HCG hormone therapy was conducted. Spawning rate for female and male mutants that received hormone therapy were improved to 80.0% and 88.9%, respectively, which were not different from controls (P=1.000, P=0.737), 71.4% without LHRHa injection and 66.7% with LHRHa injection. F_1 embryo mean hatch rate for both female and male mutants that received hormone therapy were improved to 35.0%, which were not different from controls (P=0.259, P=0.351), 32.3% without LHRHa injection and 56.5% with LHRHa injection (Table 6).

Table 6. The spawning rate of P_1 mutants and the mean embryo hatch rate of F_1 channel catfish (*Ictalurus punctatus*) before and after 1200IU human chorionic gonadotropin (HCG) hormone therapy in 2016 and 2017. Two controls were used, including non-injected control (nCTRL) and injected control (iCTRL) with 90 μg kg^{-1} luteinizing hormone releasing hormone analog (LHRHa) implantation. Hatch rate is the number of live embryos in

each family divided by the total number of embryos in each family and multiplied by 100. Mean hatch rate data was presented as the mean ± standard error (SEM).

Treatment	Sex	Spawning rate and the mean of the embryo hatch rate							
		Before hormone therapy (2016)				After hormone therapy (2017)			
		Fish N	Spawned fish N	Spawning rate (%)	Mean hatch rate ± SEM (%)	Fish N	Spawned fish N	Spawning rate (%)	Mean hatch rate ± SEM (%)
FSH	F	3	1	33.3	1.0	10	8	80.0	35.0±0.14
	M	5	2	40.0	0.5±0.05	9	8	88.9	35.0±0.14
nCTRL	F and M	5	3	60.0	74.3± 0.02	7	5	71.4	32.3±0.20
iCTRL	F and M	/	/	/	/	3	2	66.7	56.5±0.34

Notes:
Spawning rate was not significantly different between P_1 mutants and wild type control before and after hormone therapy in 2016 and 2017 respectively (Student's *t* test, *P*=1.000, *P*=0.737).
Hatch rate was significantly different between FSH gene edited group and control group before hormone therapy in 2016 (Student's *t* test, *P*=0.001).
Hatch rate were not significantly different between FSH gene edited group and non-injected control group, and between FSH gene edited group and injected control group after hormone therapy in 2017 (Student's *t* test, *P*=0.259, *P*=0.351).

3.3 Analysis of Mutation Efficiency of F1 Generation

There are total one F_1 family obtained in 2016 (M♀×Wt♂), and seven F_1 families obtained in 2017 (M♀×M♂). The mutations were successfully inherited to the offspring, with inheritance rates ranged from 45.5% to 64% (Table 8). Surveyor assay results showed multiple bands for mutants (Fig. 12).

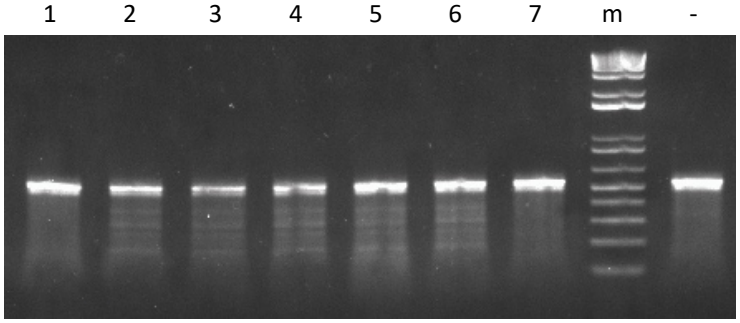


Figure. 12 Identification of edited follicle stimulating hormone (FSH) gene in F_1 channel catfish (*Ictalurus punctatus*) using Surveyor mutation detection assay. "-" indicates the negative control with wild type template; "m" indicates 1kb DNA ladder; 2, 3, 4, 5, 6 are channel catfish with mutation; 1, 7 are channel catfish without expected mutation.

3.4 Pleiotropic Effects

Pleiotropic effects were evaluated based on behavior, hatch rate, survival rate and bodyweight. For P_1 embryo and 6-months fingerlings, hatch rate and survival rate were not significantly different between the plasmids electroporated group and the control group (P=0.760, P=0.602). For 4-year-old P_1 fish, survival rate was not different between mutants and non-mutants in circulating system (P=1.000), while in the pond, survival rate of mutants are higher than non-mutants (P=0.023) (Table 7).

Table 7. Comparisons of the embryo hatch rate, 6-months fingerlings survival rate, and the survival rate of 4-year-old P_1 mutant fish and their full-sibling controls cultured in recirculating systems and ponds of channel catfish (*Ictalurus punctatus*).

Genotype	P_1 embryo and 6-months fingerlings of treatment group and control group					4-year-old mutant and non-mutant P_1 fish in 2017					
						Circulating system			Pond		
Treatment or Mutants	N eggs	N embryo hatched	Hatch rate (%)	N fingerlings survived	Survival rate (%)	N fish	N fish survived	Survival rate (%)	N fish	N fish survived	Survival rate (%)
	200	80	40.0	57	71.3	42	23	54.8	37	20	54.1
Controls or Non-mutants	200	84	44	63	75.0	2	1	50.0	6	0	0

Notes:
Hatch rate and survival rate were not significantly different between treatment group and control group for P_1 embryo and 6-months fingerlings (Fisher's exact test, P=0.760, P=0.602).
Survival rate was not significantly different between mutants and non-mutants for 4-year-old P_1 in circulating system (Fisher's exact test, P=1.000). But sample size is very small for non-mutants.
Survival rate was significantly different between mutants and non-mutants for 4-year-old P_1 in pond (Fisher's exact test, P=0.023).

The adult fish with outstanding second sexual characteristics were selected for the spawning trial. Three of five mutant males and two of three mutant females did not exhibit normal reproductive behaviors. Abnormal courtship of wild type females was observed through the bottom of the transparent aquaria when they were paired with mutant males. Some wild type females, laid immature eggs without generating an egg mass. In 2017, 2 of 5 (40.0%) mutant males and 1 of 3 (33.3%) mutant females exhibited normal reproductive behavior after 1200IU of HCG hormone therapy.

For the offspring, body weight was not different between mutants and non-mutants in six families (P=0.807, P=0.827, P=0.156, P=0.009, P=0.585, P=0.373, P=0.223, P=0.578), except for one family of 2017 FSH-3 had lower body weight for non-mutants (P=0.009). Survival rate was not different among F1 families except one family of 2017 FSH-5 (P=0.110). A low dissolved oxygen accident happened in one family of 2017 FSH-5. The mutation rate for this family is 56.7%. Thirty dead fish were collected and analyzed in one tank. Mutation rate was not different between the family of 2017 FSH-5 and the dead diseased fish in this family (P=0.475) (Table 8).

Table 8. Survival rate, mutation rate and mean body weight of 1 family of 1.5-year-old F_1 offspring spawned in 2016, 7 families of 1-year-old F_1 offspring and their controls spawned in 2017 of channel catfish (*Ictalurus punctatus*). Mean body weight data was presented as the mean ± standard error (SEM).

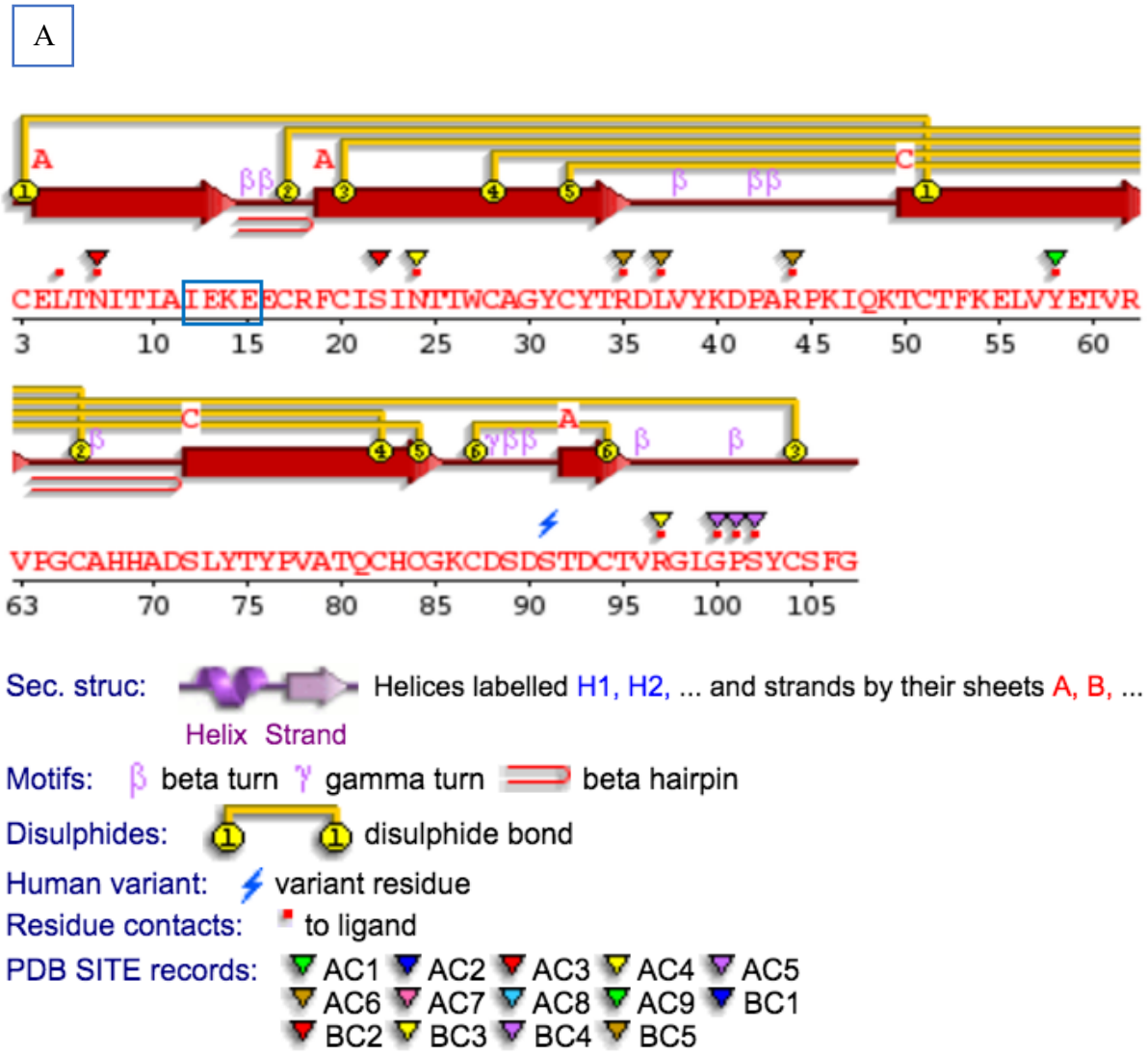
Different Families of F_1 offspring	Survival rate calculation			Mutation analysis			Mean body weight (g) ± SEM of mutant fish	Mean body weight (g) ± SEM of non-mutant fish
	N fish	N fish survived	Survival rate (%)	N fish sampled	N mutant fish	Mutation rate%		
2016 FSH	250	222	88.9	30	19	63.3	36.4±2.13	35.6±2.21
2017 FSH-1	22	20	91.0	20	11	55.0	30.2±4.76	28.6±5.26
2017 FSH-2	300	279	93.0	30	15	50.0	9.5±0.55	10.9±0.81
2017 FSH-3	24	20	83.3	25	16	64.0	15.2±1.09	22.8±2.19
2017 FSH-4	300	266	88.7	21	10	47.6	24.6±3.22	26.6±1.89
2017 FSH-5	300	168	56.0	30	18	60.0	12.8±0.82	11.9±0.62
2017 FSH-5 dead fish	/	/	/	30	18	60.0	16.67±0.811	17.67±1.509
2017 FSH-6	300	259	86.3	28	13	46.4	18.3±1.48	16.2±0.78
2017 FSH-7	300	269	90.0	22	10	45.5	8.4±0.70	9.3±1.41

Notes:
Body weight was not significantly different between mutant fish and non-mutant fish in seven F_1 families, including 2016 FSH, 2017 FSH-1, 2017 FSH-2, 2017 FSH-4, 2017 FSH-5, 2017 FSH-6, 2017 FSH-7 (Student's *t* test, P=0.807, P=0.827, P=0.156, P=0.585, P=0.373, P=0.223, P=0.578).
Body weight was significantly different between mutant fish and non-mutant fish in the family 2017 FSH-3 (Student's *t* test, P=0.009).
Survival rate was not significantly different among F1 families except 2017 FSH-5 (Student's *t* test, P= 0.110).
Mutation rate was not significantly different between 2017 FSH-5 family and the dead diseased fish in this family (Fisher's exact test, P=0.475).

3.5 Sequence Structure Analysis

FSH protein was composed of 132 amino acids. The target alignment shows significant similarity with a cutoff E-value of 2e-30 between FSH and Human Follicle Stimulating Hormone Complex Chain B. The predicted secondary protein structure of FSH protein was predicted using PDBsum, which including 7 β-turns and 1 γ-turns (Fig. 13A). TALEN targets the 4 amino acids of "IEKE", where a beta hairpin motif exists. Meanwhile, the tertiary structure of FSH was

identical to 1hcnB with C-score of -0.27, and the image was colored by rainbow from N to C terminus (Fig. 13B).



B

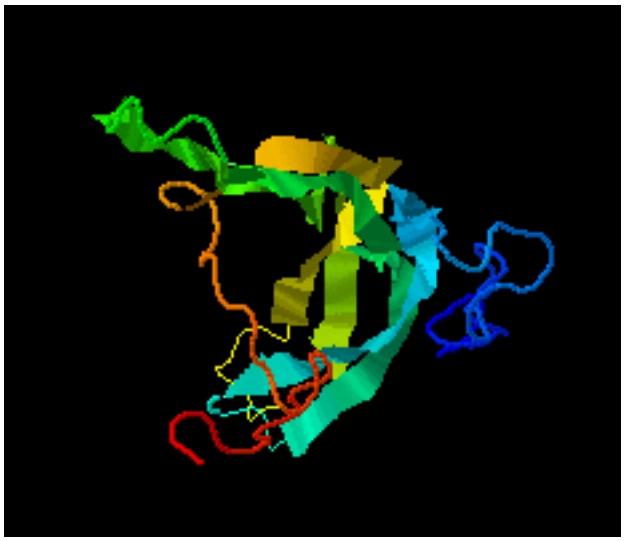


Figure 13. Prediction and analysis of follicle-stimulating hormone (FSH) protein structure. **(A)** Secondary structure of FSH was predicted using PDBsum Generate, which has 10 β-turns and 1 γ-turns. Transcription activator-like effector nuclease (TALEN) targets the 4 amino acids of "IEKE". Blue box represents the targeted amino acids. **(B)** Three-dimensional structure of FSH was predicted using I-TASSER server, which is identical to 1hcnB with C-score of -0.27, and the image was colored by rainbow from N to C terminus.

4 Discussion

Different types of mutations were found at the targeted site of FSH gene, including base deletions, substitutions, and insertions, which were successfully inherited to F_1 offspring. Indels have possibility to induce a premature stop codon to suppress the function of FSH. In addition, the base substitution could introduce a stop codon, or change a codon to one that encodes a different amino acid. The cleavage site is predicted as a β hairpin motif. Therefore, a single change of one amino acid could change the protein secondary and 3d structure and then block the

function. The spawning rate of P_1 mutants and their eggs hatch rate were suppressed. HCG hormone therapy restored fertility of P_1 mutants. No obvious pleiotropic effects were found in seven families of F_1 offspring, but only one family of F_1 offspring had lower body weight for mutants, which may be caused by the small sample size. Four-year-old P_1 mutants have higher survival rate.

TALENs generally are designed to target genes with high specificity and low cytotoxicity (Liu et al. 2014, Mussolino et al. 2014, Dreyer et al. 2015, Veres et al. 2014). For the P_1 generation, both the embryo hatch rate and fry survival rate of the TALEN treatment group was not different with the control group, which suggests that the TALEN plasmids and the nucleases had high specificity and only targeted the FSH gene with little if any off-target mutations. After 4 years, no differences in survival were found between P_1 mutant fish and their full-sibling controls cultured in recirculating systems further confirming this conclusion. P_1 mutants even had higher survival rate in pond compared with their full-sibling controls.

The TALENs target sites were designed to be located at the β subunit of FSH gene, which confers the physiological specificity of the hormone, to suppress the translation of FSH. On the other hand, as part of a multimeric complex, an abnormal variant might act as a "poison subunit" rendering the whole complex nonfunctional and that such a mutant would behave genetically as an antimorph, disrupting proteasome activity in a dose-dependent manner (Saville and Belote 1993, Schweisguth 1999). The maximum levels of the synthesis and secretion of FSH correspond to the stage of vitellogenesis in the annual reproductive cycle in salmon (Dolomatov et al. 2012). In the female, FSH has effect on ovarian functions through membrane receptors on the granulosa and theca cells. In fish, the presence of their specific binding sites in the ovary has been demonstrated in the amago salmon (*Oncorhynchus rhodurus*) (Kanamori et al. 1987) and

murrel (*Channa punctatus*) (Uddin and Bhattacharya 1986). The knockout of FSH gene should restrain the synthesis and release of pituitary gonadotropin, followed by the repression of the secretion of steroid hormones, including estradiol and testosterone.

However, despite the numerous reports on FSH in teleosts in the past 20 years, we are still far from understanding the functions of FSH in controlling fish reproduction. In salmonids, it has been proposed that FSH may be responsible mainly for ovarian growth, whereas LH is important in inducing final oocyte maturation and ovulation (Breton et al. 1998, Prat et al. 1996, Weil et al. 1995).

In zebrafish (*Danio rerio*), the hormone-specific β-genes of both FSH and LH were knocked out separately or together using TALEN. For FSH-deficient zebrafish, a significant delay of ovary, testis development and puberty onset were observed. However, they are still fertile in both sexes. Also, FSH seemed to play a role to maintain the female status, because sexual reversal was observed in the FSH deficient zebrafish. The double mutation of the FSH and LH genes led to all males, although the development of the testis was significantly delayed (Zhang et al. 2015). Our results in channel catfish are different from the results in zebrafish. Some FSH deficient channel catfish (*Ictalurus punctatus*) with outstanding secondary sexually characteristics cannot spawn with abnormal reproductive behaviors. The F_1 embryo from FSH deficient P1 females or males had low hatch rate without HCG hormone therapy, while both spawning rate and hatch rate increased after hormone therapy.

However, whether this is also the situation in other groups of teleosts such as cyprinids remains to be elucidated, especially when we consider the fact that the specificity of FSH receptors varies significantly among different species. FSH bound only to the type I receptor (now termed FSH receptor) with limited interaction with the type II receptor (LH receptor) in

coho salmon (*Oncorhynchus kisutch*) (Yan et al. 1992). In the African catfish (*Clarias gariepinus*), both purified and recombinant LH and FSH could elevate intracellular cAMP level (Vischer et al. 2003). In an Indian carp (*Labeo rohita*), the situation is even more complicated. The two gonadotropin receptors purified from the ovarian follicles recognized both salmon FSH and LH in receptor binding assays, albeit with preference for their cognate ligands (Basu et al. 2002).

Glycoprotein hormone receptors (GPHRs) contain 3 subfamilies, including luteinizing hormone/choriogonadotrophin receptor (LHCGR), follicle-stimulating hormone receptor (FSHR) and the thyroid-stimulating hormone receptor (TSHR) (Ascoli et al. 2002, Troppmann et al. 2013). In equine, equine chorionic gonadotropin (eCG), a member of the glycoprotein family of hormones along with LH, FSH and thyroid-stimulating hormone, has been used for control reproduction in cow (De Rensis and López-Gatius 2014). In fish, human chorionic gonadotropin (HCG) has been successfully used for many years to induce final maturation and spawning. HCG and FSH/LH combination were equally effective in inducing oocyte maturation and preventing onset of atrophy before completion of vitellogenesis in grey mullet (*Mugil cephalus L.*) (Shehadeh et al. 1973). In perch (*Perca fluviatilis*), similar situations were observed after hormonal treatment of common carp, *Cyprinus carpio*, pituitary extract and HCG (Kucharczyk et al. 1996). Both LH and HCG interact with the same receptor (LH/HCG receptor), and HCG and LH have both been used therapeutically to stimulate events that are caused by the interaction of either hormone with the LH/HCG receptor. The LH/HCG receptor is a large cell-surface glycoprotein with the characteristic structure that makes it a member of the superfamily of G protein-coupled receptors (Kido et al. 1996). FSH stimulates the growth and proliferation of granulosa cells through its interaction with its specific surface receptors (Filicori et al. 2005).

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Ⅲ Gene Editing of the Luteinizing Hormone Gene and Hormone Therapy to Control the Reproduction in Channel Catfish, *Ictalurus punctatus*

Abstract

Fish sterilization technology could minimize the environmental risk from escapees or released genetically engineered individuals on natural population. Critical reproductive genes mutation is one of the approach to achieve fish sterilization. Luteinizing hormone (LH) gene plays an important role in reproduction. Zinc finger nucleases (ZFNs) plasmids were delivered targeting the LH gene into fertilized eggs through double electroporation to sterilize channel catfish (*Ictalurus punctatus*). The cleavage efficiency was 38.9% located around 60 bp upstream of the expected site in P_1 fish. Channel catfish females carrying the mutated LH gene were sterile, as confirmed by DNA sequencing and mating experiments. None of the mutant males could spawn when they were 3-year-old, while 1 of 4 mutant males fertilized the eggs with egg hatch rates of 1% when they were 4-year-old. Spawning rate and eggs hatch rate were significantly different between mutants and wild type control. Human chorionic gonadotropin (HCG) hormone therapy increased the spawning and hatch rates for mutants, which were not different from controls (P>0.05). No integration of the plasmids and off-target effects in terms of growth rate, survival and appearance were detected in mutants.

1 Introduction

There are more than 3,700 species of catfishes, *Siluriformes* (Nelson et al. 2016). Members of the family *Ictaluridae*, especially the genus *Ictalurus* and their hybrids are extensively cultured worldwide and is the leading aquaculture species in the United States, accounting for over 60% of US aquaculture production (Hanson and Sites 2014). Catfish are valuable worldwide as their basal phylogenetic position and an important source of dietary protein (Liu et al. 2016). However, domestic catfish production shrank by more than 50% since 2003 (Hanson and Sites 2014), because of competition from Asia, increased feed and labor costs and the control of fish diseases (Wagner et al. 2012).

Permanent genetic alterations to brood stocks by selective breeding and transgenesis can make major improvements in performance in channel catfish (Dunham 2011). The genome sequence of channel catfish has been reported and validated by genetic mapping of 54,000 SNPs and annotated with 26,662 predicted protein-coding genes, which provided crucial resources for biological studies (Liu et al. 2016). However, the transgenic technology has the capability to induce novel phenotypes (Dunham and Liu 2006). Effective biological containment can be best achieved by sterilization through genome editing that can directly eliminate the potential for reproductive interaction between wild and cultured stocks.

Genome editing technologies can be used to make specific changes at targeted genomic sites to introduce an immediate change in a single generation, which hold great promise for improving aquaculture (Abdelrahman et al. 2017). Zinc finger nuclease (ZFN) is a site-specific gene editing technology, utilizing a FokI endonuclease domain and a DNA binding domain to induce double-strand breaks (DSBs) to specific DNA sites (Baker 2012). Usually DSBs are repaired by non-homologous end joining (NHEJ) and typically result in small insertions or deletions that will disable the gene because of the change or shift of the translational reading

frame (Stoddard 2011). Induction of sterility using gene edited fish containing engineered genes capable of blocking normal reproductive development has considerable potential for allowing effective reproductive containment of genetically altered fish.

Sex determination, reproduction and reproductive strategies are quite diverse in fish, including genotypic sex determination, male heterogamety, female heterogamety, environmental sex determination, sequential hermaphroditism and polygenic sex determination (Bachtrog et al. 2014). Additionally, the major sex determining genes differ among fish species, are evolving (Pan et al. 2016) and can even involve transposable elements (Schartl et al. 2018). Until recently, gonadal maturation in teleost fishes is widely believed to be principally controlled by hypothalamus-pituitary-gonad (HPG) axis. The central regulators along this axis are the gonadotropic hormones, FSH and LH, both synthesized and secreted by the pituitary gland. Their coordinated action enables fine-tuning of ovarian and testicular function.

LH belongs to the glycoprotein hormone family, which is a heterodimer consisting of non- covalently linked and subunits. The subunit is shared by all members of the family, while the subunit is structurally distinct and hormone specific, determining the specificity of the hormones (Pierce and Parsons 1981). Within a given fish species all α subunits are identical while the different β subunit confers the physiological specificity of the hormone (Liu et al., 2001).

It is known that during secondary oocyte growth, plasma levels of FSH are elevated while the circulating levels of LH are extremely low or non-detectable. At this stage, the FSH receptor has been shown to be present in both theca and granulosa cells. However, during final oocyte maturation (FOM), plasma levels of LH increase as FSH levels decline in parallel with the appearance of the LH receptor in granulosa cells (Miwa et al. 1994, Yan et al. 1992). In

addition, during the transition from vitellogenesis to FOM, the changes in circulating levels of FSH and LH occur concomitantly with changes in the production of two major ovarian steroids: estradiol-17b (E_2) and 17a,20b-dihy-droxy-4-pregnen-3-one (17,20b-P), respectively (Goetz et al. 1987, Nagahama et al. 1995). It is possible that FSH and LH play important roles in regulating the steroidogenic shift in the ovary, which determines the transition from the vitellogenic state to the maturational state. However, the role that FSH and LH play in regulating the changes in steroid production that take place in the teleost ovary during the process of FOM remain to be determined.

The objectives of this study were to use ZFNs technology to block the function of LH gene on the genomic level, leading to sterility in channel catfish (*Ictalurus punctatus*), and then restore the fertility of gene edited sterile channel catfish through hormone therapy. Specifically, ZFNs plasmids targeting the LH gene were delivered into eggs and fertilized eggs with double electroporation technique. Fish mating experiments were utilized to evaluate the reproductive capability of mutant catfish. LHRHa, carp LH and human chorionic gonadotropin (HCG) were applied to reinstate the fertility of gene edited sterile channel catfish. Mutation analyses were employed on F_1 fish to verify the heredity of mutations. Hatch rate, survival rate and body weight were measured on F_1 fish to assess the pleiotropic effects.

2 Materials and Methods

2.1 Construction of Plasmid

Zinc finger nucleases (ZFNs) plasmids were designed and assembled by Sigma-Aldrich company (St. Louis, MO). All the plasmids were driven by the cytomegalovirus (CMV) promoter and the T7 promoter, followed with the zinc finger domain, FolkI domain and the

kanamycin resistant element (Fig. 14). The ZFNs target sites were designed to be located at the β subunit of LH gene (GenBank Accession No. AF112192.1), which confers the physiological specificity of the hormone. Each LH comprised an 16 bp (5'-CACAGAAACAGTCTCA-3') left DNA binding site, or an 18 bp (5'-GGTTCGCAGTGTGGCA-3') right DNA binding site.

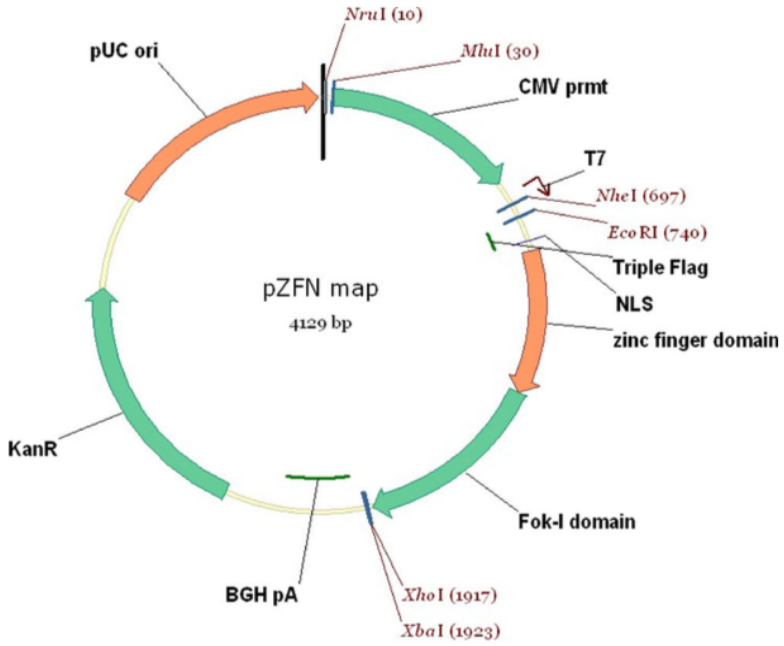


Figure 14. Schematic representation of zinc finger nucleases (ZFNs) plasmid structure targeting luteinizing hormone (LH)gene of channel catfish (*Ictalurus punctatus*). *CMV prmt* cytomegalovirus promoter, *NLS* nuclear localization signal, *BGH pA* bovine growth hormone and polyadenylation signal, *KanR* kanamycin resistance gene.

2.2 ZFN Plasmid Replication, Extraction and Dilution

The ZFN plasmids were transformed into One Shot® TOP10F′ Competent Cells (Invitrogen, Grand Island, NY), following the transformation procedures from the manufacturer. One hundred μL of each transformation mix vial was spread on the LB agar plate with 100 μg ml^{-1} ampicillin separately. A single colony was picked from each plate and then cultured in 400

mL LB broth with 100 μg ml^{-1} ampicillin separately. Then the LH plasmids were extracted with the IsoPure Plasmid Maxi II Prep Kit (Denville, Holliston, MA). DNA agarose gel electrophoresis and spectrophotometry were then used to test the quantity and quality of the ZFN plasmids. Equal amounts of both left and right ZFN plasmids were mixed together. They were diluted with 2 mL saline (0.9% NaCl) to obtain a 25 μg ml^{-1} mix solution and diluted with 5 mL TE buffer (5mM Tris-HCl, 0.5M EDTA, pH=8.0) to collect a mix solution with the same concentration, respectively.

2.3 Experimental Brood Stock and Gametes

Sexually mature females of Kansas Random strain of channel catfish (*Ictalurus punctatus*) were selected and implanted with luteinizing hormone releasing hormone analog (LHRHa) (Reproboost® Implants, Center of Marine Biotechnology, Columbus Center, Maryland, USA) with a dose of 90 μg kg^{-1}. All showed outstanding secondary sexual characteristics. Eggs of two anesthetized ovulating females were hand stripped into dried stainless steel pie pans greased with vegetable shortening. Two-hundred eggs were collected for each experimental group and control group. One Kansas Random strain male and one Auburn-Rio Grande strain male (Dunham and Smitherman, 1983) were euthanized. Mixed testes were placed into a mesh strainer and macerated manually into saline (0.9% NaCl) to collect sperm.

2.4 Fertilization, Electroporation and Incubation

The first electroporation was conducted on the collected sperm homogenate with the ZFN saline solution using a Baekon 2000 macromolecule transfer system (Baekon, Inc., Saratoga, CA) with parameters of 6kV, 27 pulses, 0.8s burst, 4 cycles and 160μs (Su et al. 2013). Then two

hundred prepared eggs were artificially fertilized with the electroporated sperm. After one hour, the embryos were gathered and immersed with the ZFN TE buffer solution for ten minutes. Then the second electroporation was conducted with the same procedure. The control group was generated following the same protocols, but with the absence of ZFN plasmids. Embryos were then moved into 10 L tubs with Holtfreter's solution containing 10 ppm doxycycline and incubated statically until hatch. Dead embryos were removed daily before Holtfreter's solution was changed.

2.5 Sample Collection and Mutation Analysis for P_1 generation

Embryos were cultured for 6-7 days until hatch in tubs at 27°C and then transferred into a recirculating system. After 6 months, the genomic DNA was extracted from the pelvic fin and barbel samples. Samples were digested with cell lysis buffer and proteinase K following the protocols by Cheng et al. (2014). DNA agarose gel electrophoresis and spectrophotometry were then used to test the quantity and quality of the genomic DNA. Then Roche Expand High FidelityPlus PCR System (Roche, Indianapolis, IN) was used to amplify the channel catfish LH gene on these DNA samples. The following primers were utilized: forward sequence 5'-AGGATGTCAGTGCCAGCTTC -3'; reverse sequence 5'- CTTGGAGTAAATGGACTCGTTG -3' (Table 1), and the amplification procedure was: initial denaturation for 2 min at 94°C, followed by 29 cycles of 94°C for 30s, 60°C for 30s and 72°C for 40s, and the final elongation for 10min at 72°C.

Gene mutations were detected using Surveyor® Mutation Detection Kit for Standard Gel Electrophoresis (Integrated DNA Technologies, Coralville, IA) (Qin et al. 2015). The key component of the kit is Surveyor Nuclease, a member of the CEL family of mismatch-specific

nucleases derived from celery, which can be used to perform a highly specific cleavage on the 3' side near the mutation site of the two strands of the heteroduplex DNA. The recognized mutant forms include single base or multiple base insertions, deletions or mismatches (Qiu et al. 2004). Surveyor Nuclease has been a powerful and repeatable tool for mutation testing. It has been used to detect mutations and polymorphism in human, mammal, bacteria, and plant genomes (Pimkin et al. 2004; Tsuji and Niida, 2008). The processes were as follows: first, the PCR products was hybridized using a PCR machine to form a heteroduplex using a thermal cycler as 95°C 10min, 85°C 1min Ramp 2°C/s, 75°C 1min Ramp 0.3°C/s, 65°C 1min Ramp 0.3°C/s, 55°C 1min Ramp 0.3°C/s, 45°C 1min Ramp 0.3°C/s, 35°C 1min Ramp 0.3°C/s and 25°C1 min Ramp 0.3°C/s; then a nuclease reaction system, which included DNA hybridization system, 150mM $MgCl_2$, 5×PCR buffer, Surveyor enhancers S and Surveyor nucleases were established and incubated in a PCR machine under 42°C for 1 hour; finally, the 2% UltraPure Agrose-1000 (Invitrogen, Grand Island, NY, USA) was used to perform electrophoresis to test the mutation.

2.6 TA Clone and Sequencing

To confirm the presence of the mutations, Roche high fidelity PCR amplification targeted LH gene was performed with the sample DNA. Then TOPO® TA Cloning® kit was used to set up a 6μL TOPO cloning system, which included 4μL amplified DNA product, 1μL salt solution and 1μL TOPO vector. The system was mixed and incubated under room temperature (22-23°C) for 5min, followed by competent cell transfection. First, 2μL of the cloning system was added into one vial of One Shot®TOP10F'competent cells and put on the ice for 30min. Then 42°C heat shock was implemented using PCR machine for 30s. After that, the tube was placed on ice immediately. The next, 250μL LB (Tryptone 10g L^{-1}, Yeast extract 5 g L^{-1}, NaCl 10 g L^{-1},

Agarose 10g) broth was mixed together in a 1.5ml tube and placed in the 37°C shaker and shaken at 200rpm for 1 hour. Next, 50μL, 80μL, and 100μL mixtures were spread on the LB (Tryptone 10 g L^{-1}, Yeast extract 5 g L^{-1}, NaC l10 g L^{-1}, Agarose 10g) medium and placed in the 37°C incubator overnight. Ten monoclonal colonies were picked from each plate, mixed with 400ml LB (Tryptone 10 g L^{-1}, Yeast extract 5 g L^{-1}, NaCl 10 g L^{-1}, Agarose10g) broth and placed in the 37°C incubator overnight. The amplified bacterial solution was dispensed into a 96-wells plate and sent to the Eurofins Genomics Company (Louisville, KY, USA) for sequencing. Finally, sequencing results were analyzed using T-Coffee tool (Notredame et al. 2000).

2.7 Plasmid Integration Inspection

PCR was conducted to determine if plasmids were integrated into the genome or persisted in the cytoplasm. Two pairs of primers targeting LH gene were designed to detect the plasmid DNA in mutant fish. The amplification regions were vector backbone and ZFN domain respectively. They were amplified using the primers as shown in Table 9. The amplification procedure was as follows: initial denaturation for 2 min at 94°C, followed by 29 cycles of 94°C for 30s, 60°C for 30s and 72°C for 40s, and the final elongation for 10min at 72°C. The results were generated using electrophoresis.

Table 9. Primer sequences used for the amplification of luteinizing hormone (LH), vector backbone and zinc finger nuclease (ZFN) domain in channel catfish (*Ictalurus punctatus*).

Targeting Site		Sequence
LH	Forward	5'-AGGATGTCAGTGCCAGCTTC-3'
	Reverse	5'-CTTGGAGTAAATGGACTCGTTG-3'
Vector backbone	Forward	5'-GTGTACGGCTACAGGGGAAA -3'
	Reverse	5'-TTGGGGTTGAGGTGCTTATC -3'
ZFN domain	Forward	5'-CGTGACCGAGTTCAAGTTCC-3'
	Reverse	5'-AAGTTGATCTCGCCGTTGTT-3'

2.8 Reproductive Evaluation

The fingerlings were transferred to 0.04- hectare pond or a recirculating system when they reached 15-20cm for further growth and maturation. When LH mutant channel catfish reached 2 years old, 4 mutant (M) males and 4 mutant females were paired in individual aquarium to mate (M♀×M♂, M♀×M♂) without hormone injection. Meanwhile, 4 pairs of wild type (Wt) channel catfish also were paired in individual aquaria as control without hormone injection. When 3 years old, 11 pairs of putative LH-mutated channel catfish were paired in aquaria (M♀×M♂, M♀×M♂). Fifteen control pairs were utilized at the same time. In each case, they were given 14 days to spawn naturally. After that, they were given carp pituitary extract (CPE) hormone therapy to restore the fertility.

When LH mutant channel catfish reached 4 years old in 2016, there were 4 mutant males and 6 mutant females selected to mate with wild type (Wt) females and males (Wt♀×M♂, M♀×Wt♂). There were five pairs of controls channel catfish were selected for natural spawning without hormone injection. For testing the fertility of mutant males, only the paired wild type females were implanted with 75 μg kg^{-1} LHRHa for induced spawning. Then the wild type

females that did not spawn within 6 days were implanted again with 90 μg kg^{-1} LHRHa. If the females did not spawn after two implantings, they were replaced by other wild type females and the hormone implantation for laying eggs. For testing the fertility of mutant females, the pairs were given 15 days to spawn naturally with the wild type males and without hormone injection.

All fish were paired in 48cm×36cm×21cm aquaria with constant water flow and compressed air for aeration. All fish showed outstanding secondary sexual characteristics. The sexually mature males had well-developed head muscles and dark mottle in the lower jaw and abdomen. The sexually mature females exhibited fuller, rounder abdomen and reddening genital area compared to immature females.

2.9 Hormone Therapy

Two-year-old mutant fish were not given hormone therapy. Three-year-old mutant fish were injected with a priming dose of 2 mg kg^{-1} of carp pituitary extract (CPE) followed by a resolving dose of 6 mg kg^{-1} CPE 12 h later (M♀×M♂). When any 3-year-old fish did not respond to the CPE injections after 4 days, they were given two more injections of CPE at 2 mg kg^{-1} every 3 days. For 4-year-old mutant fish, there were 3 mutant males and 3 mutant females mated with each other and injected with 60μg kg^{-1} LHRHa and 400IU/kg carp LH (M♀×M♂).

For 5-year-old mutant fish, 3 mutant males and 5 mutant females with outstanding secondary sexual characteristics were selected to mate with wild type fish (Wt♀×M♂, M♀×Wt♂). Both females and males were injected with 133ug kg^{-1} LH and 20ug kg^{-1} liquid LHRHa. If females and males did not spawn within 7 days, they were injected again with 400ug kg^{-1} carp LH and implanted with 80ug kg^{-1} LHRHa. If females and males did not spawn within 9

days, they were injected again with 1200IU kg^{-1} human chorionic gonadotropin (HCG). Five pairs of controls fish were selected for natural spawning without hormone injection.

2.10 F_1 Fish Culture

When egg masses were obtained, they were placed in baskets in hatching troughs with constant water flow and aeration. Calcium chloride solution was continually dripped into the trough to ensure 40-50ppm hardness (Steeby, 2005). Eggs were gently agitated with a paddlewheel beginning 2 hours after spawn collection. The egg masses were prophylactically treated with 100ppm formalin or 32ppm copper sulfate every 8 hours to avoid fungus (Small et al. 2006). The treatments were terminated 12 hours before hatch.

Catfish embryos began hatching in 7 days with a water temperature between 26-28℃. They consumed their yolk sac and began swim up stage 3 days post hatching. They were then fed artemia (Brine Shrimp Eggs, Carolina Biological) three times a day and stocked into a recirculating system with densities of 1000 fish per 90L tank and 100 fish per 60L aquaria of each family. After one month fry were fed Purina® AquaMax® Fry Powder (Purina, St. Louis, MO) three times a day. As fry grew they were then fed Purina® AquaMax® Fry Starter 100. After further growth fry were moved to 90L tank at a stocking density of 500 fish per tank and fed Purina® AquaMax® Fry Starter 200 and 300 three times a day.

2.11 Sample Collection and Mutation Analysis for F_1 generation

When 2017 spawned F_1 fish were 1-year-old, ventral fin samples were collected from 20-30 fish in each family. Surveyor mutation analysis were conducted. The procedures were the same as described above.

2.12 Pleiotropic Effects

For P_1 fish, survival rate for embryos, 6-month-old fingerlings and 4-year-old adult fish was measured. For F_1 fish, survival rate and body weight for F_1 1-year-old fish were measured. The reproductive behavior was observed clearly through the bottom of overhead aquariums.

2.13 Statistical Analysis

Statistical analysis of mutation rate, survival rate, hatch rate and body weight were performed with R studio software (RStudio Inc, Boston, MA). Mutation rate, survival rate, and hatch rate were compared utilizing Fisher's exact test in case of small sample size. Student's *t*-test was performed to compare hatch rate and spawning rate between treatments and controls for P_1 fish. Student's *t*-test was also applied to compare survival rate and mutation rate between F_1 families before and after hormone therapy, and compare body weight in each F_1 families. Shapiro- Wilk's test was used to test the normality of the data. Comparisons of statistical significance were set at $P<0.05$, and all data were presented as the mean ± standard error (SEM).

2.14 Sequence Structure Analysis

LH mRNA sequences were retrieved from the nucleotide NCBI database (https://www.ncbi.nlm.nih.gov/). Amino acid sequences of LH protein were translated using ExPASy software (https://web.expasy.org/translate/). Sequences of LH protein was used as queries to BLAST against protein data bank (PDB) database with a cutoff E-value of $1e^{-10}$. If it shows significant similarity with query sequence, then the secondary structures of LH protein was predicted using PDBsum software (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl?pdbcode=index.html). If it doesn't show significant similarity with

query sequence, then the secondary structures of LH gene was predicted using Pole Bioinformatique Lyonnais (PBIL) server (https://prabi.ibcp.fr/htm/site/web/home). The three-dimensional protein structure was predicted using the I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). Only models with the C-score between 2 and -4 were considered. C-score is a confidence score for estimating the quality of predicted models. It is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is typically in the range of [-5, 2], where a C-score of higher value signifies a model with a high confidence and vice-versa. The visualization of predicted three-dimensional structures was performed using the Jmol software. The predicted model was downloaded from I-TASSER as a PDB file. The predicted secondary protein structure based on the predicted 3D model was displayed using the PDBsum server and compared with the result from PBIL.

3 Results

3.1 Analysis of Mutation Efficiency and Plasmid Integration of P_1 Generation

In the previous research, the targeted mutagenesis have been detected in 7 of 18 6-month-old P_1 fingerlings for a 38.9% mutation rate. Surveyor assay were conducted and 3 bands were detected through electrophoresis (Fig. 15). These mutations were in the β subunit region of LH gene, which confers the physiological specificity of the hormone, and all located at around 60bp upstream of the proposed target site, but were still within the open reading frame (ORF). Different types of mutation were found, including base deletion, substitution and insertion. These mutations could induce a premature stop codon or alter the structure of LH protein to block the gene function (Fig. 16 and Fig. 17). Plasmid integration was inspected through PCR. Neither

vector backbone nor ZFNs domain was amplified in mutants, which verified none of the fingerlings carried the exogenous DNA (Fig. 18) (Qin 2015, Qin et al. 2016).

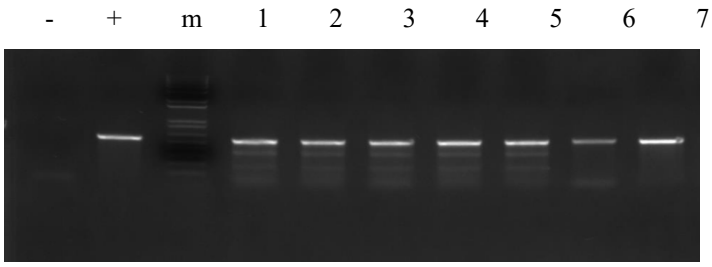


Figure 15. Identification of edited luteinizing hormone (LH) gene in P_1 channel catfish (*Ictalurus punctatus*) using CEL-I mutation detection assay. The "-" indicates the negative control without template. The "+" indicates the control with wild-type channel catfish DNA template; "m" indicates 1kb DNA ladder; 1-5 are channel catfish with mutation; 6 and 7 are channel catfish without mutation (Qin 2015, Qin et al. 2016).

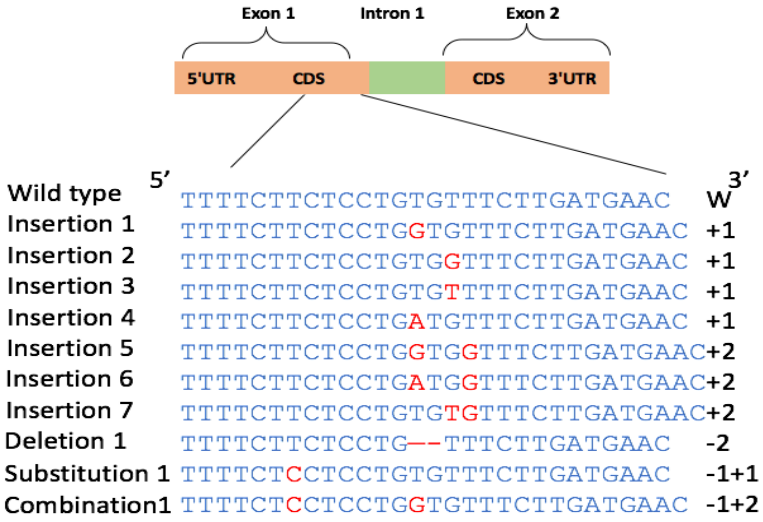


Figure 16. Sequences of luteinizing hormone (LH) gene in channel catfish (*Ictalurus punctatus*). The wild-type channel catfish LH gene sequences are shown on the top. Sequences in blue are the original sequences. Red dashes and letters indicate the deletion/insertion of nucleotides. Numbers at the end of the sequences show the number of nucleotides deleted (-) or inserted (+) in the edited LH gene. These mutations were all located at around 60bp upstream of the proposed target site, but were still within the ORF of LH β subunit gene.

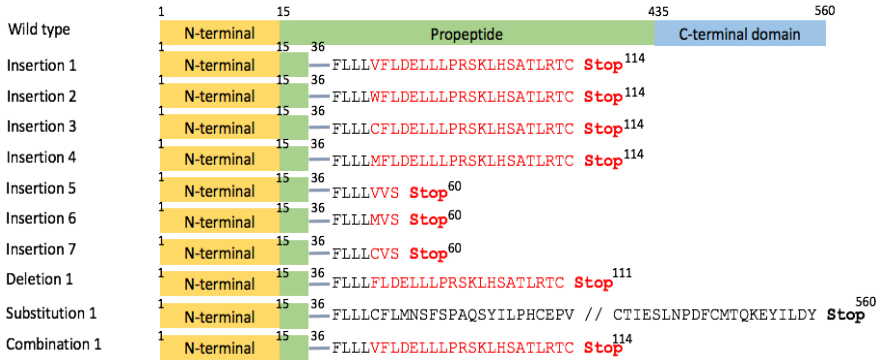


Figure 17. Predicted amino acid sequences of luteinizing hormone (LH) gene produced from the mutant channel catfish (*Ictalurus punctatus*). Amino acid sequences of the wild-type channel catfish LH gene are shown on the top. Amino acid sequences following incomplete domain were due to fame-shift reading, resulting in a premature stop (red color) codon.

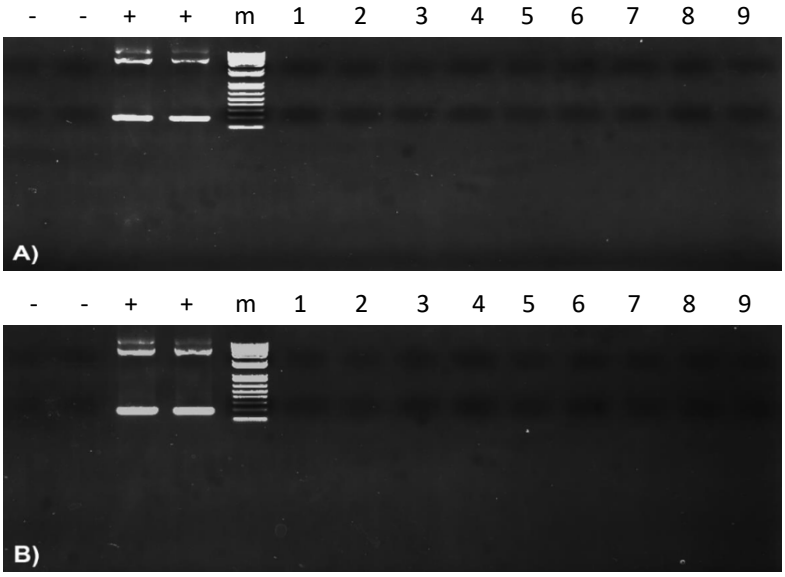


Figure 18. PCR inspection of zinc finger nucleases (ZFNs) plasmids integration into channel catfish (*Ictalurus punctatus*) genome. The left two lanes of "-" indicates the negative controls without template, the right two lanes of "-" indicates the negative controls with wild-type channel catfish as template; "+" indicates the positive controls with ZFNs left and right plasmids as template, respectively; "m" indicates 1 kb DNA ladders. Numbers represent channel catfish individuals carrying mutated luteinizing hormone (LH) gene; the same number indicates the same individual. A) & B) represent the PCR detection with different specific primers designed to amplify vector backbone and ZFNs domain, respectively (Qin 2015) (Qin et al. 2016).

3.2 Analysis of Mutation Efficiency of F_1 Generation

There were F_1 families obtained in 2017 (M♀×Wt♂, Wt♀×M♂), including 2017 LH-1, 2017 LH-2, and 2017 LH-3. The 1-year-old 2017 F_1 offspring families had 0, 50%, 50% inheritance of the mutations, respectively (Fig. 19) (Table 12). The parent banding matched with their offspring.

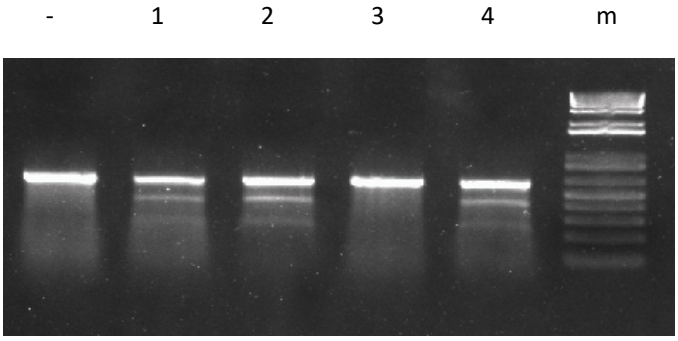


Figure 19. Identification of edited luteinizing hormone (LH) gene in F_1 channel catfish (*Ictalurus punctatus*) using CEL-I mutation detection assay. "-" indicates the negative control with wild type template; "m" indicates 1kb DNA ladder; 1, 2, 4 are channel catfish with mutation; 4 is a channel catfish without expected mutation.

3.3 Spawning Experiments and Hormone Therapy

Four pairs of 2-year-old catfish carrying the mutated LH gene were introduced to aquaria for natural spawning, but none of these catfish successfully spawned. In contrast, 3 of 4 (75%) pairs of catfish from the control group spawned naturally, and the eggs hatched. At 3 years old,

11 pairs of putative LH-mutated channel catfish did not spawn when given a 2-week opportunity to mate. A series of CPE injections did not induce any spawning. Meanwhile, 11 out of 15 (73.3%) control pairs produced fertile egg masses.

At 4 years old, 3 of 5 (60.0%) control pairs spawned with a 74.3% average hatch rate in 2016. Five of 7 (71.4%) control pairs spawned with an 32.3% average hatch rate without LHRHa hormone injection, while 2 of 3 (66.7%) control pairs spawned with an 56.5% average hatch rate with LHRHa hormone injection. P_1 mutant males had spawning rate of 25% when there was no hormone therapy with egg hatch rates of 1.0%. None of the mutant females could spawn after a series of LHRHa and carp LH hormone injections.

Different types of hormone were utilized for restoring the fertility of mutants, including LHRHa, carp LH and HCG hormone. LHRHa and carp LH failed to induce spawning, while HCG successfully induced spawning. Spawning rate for female and male mutants that received hormone therapy were 40.0% and 33.3%, respectively, which were not different from controls, 71.4% without LHRHa injection and 66.7% with LHRHa injection. F_1 embryo mean hatch rate for female and male mutants that received hormone therapy were both 50.0%, which were not different from controls, 32.3% without LHRHa injection and 56.5% with LHRHa injection (P=0.61, P=0.89) (Table 10).

Table 10. The spawning rate of P_1 and the mean embryo hatch rate of F_1 channel catfish (*Ictalurus punctatus*) before and after 1200IU HCG hormone therapy in 2016 and 2017. Two controls were used, including non-injected control (nCTRL) and injected control (iCTRL) with 90 μg kg^{-1} luteinizing hormone releasing hormone analog (LHRHa) implantation. Hatch rate is the number of live embryos divided by the total number of embryos in each family and multiplied by 100. Mean hatch rate data were presented as the mean ± standard error (*SEM*).

Treatment	Sex	Spawning rate and the mean of the embryo hatch rate							
		Before hormone therapy (2016)				After hormone therapy (2017)			
		Fish N	Spawned fish N	Spawning rate (%)	Hatch rate (%)	Fish N	Spawned fish N	Spawning rate (%)	Hatch rate (%)
LH	F	6	0	0	/	5	2	40.0	50.0±22.87
	M	4	1	25.0	1.0	3	1	33.3	50.0
nCTRL	F and M	5	3	60.0	74.3± 0.02	7	5	71.4	32.3±0.20
iCTRL	F and M	/	/	/	/	3	2	66.7	56.5±0.34

Notes:
Hatch rate were not significantly different between LH gene edited group and non-injected control group, and between LH gene edited group and injected control group after hormone therapy in 2017 (Student's *t* test, *P*=0.61, *P*=0.89).

3.4 Pleiotropic Effects

There were 47 of 200 P_1 fish were double electroporated with plasmids along a 23.5% hatch rate, which is not significantly different with controls of 42.0% (*P*=0.760). After 6 months, the fry survival rate of the ZFN LH group was 68.1%, which is not different with controls of 75.0% (*P*=0.602). After 3 and 4 years, the fish survival rate was evaluated again for mutants and their full-sibling non-mutant fish. For 3-year-old fish, the survival rate was 30.0% for mutants and 60.9% for non-mutants in the circulating system (*P*=0.141); while for 4-year-old fish, the survival rate was 75.0% for mutants and 85.0% for non-mutants (*P*=0.514) (Table 11).

In this spawning trials, no courtship behavior was exhibited before hormone therapy, except 1 mutant male mated with 1 wild type female. These fish showed outstanding secondary

sexual characteristics. Males were in prime condition and had heads with heavy muscular development, lean bodies and large, and distinct genital papillae. Females were also in prime condition with soft, protruding abdomens indicative of maximum gravidness and swollen, mucoid, and reddish genital openings. HCG hormone therapy resulted in good spawning and hatch rates for mutants in 2017. One of 3 (33.3%) mutant males and 2 of 5 (40.0%) mutant females exhibited normal reproductive behavior.

In terms of body weight, there were no significant difference between mutants and non-mutants in each F_1 family (P=0.119, P=0.184) (Table 12).

Table 11. Comparisons of the embryo hatch rate, 6-months fingerlings survival rate, and the survival rate of 4-year-old and 5-year-old P_1 mutant fish and their full-sibling controls cultured in recirculating systems of channel catfish (*Ictalurus punctatus*).

Genotype	P_1 embryo and 6-months fingerlings of treatment group and control group					4-year-old mutant and non-mutant P_1 fish in 2016			5-year-old mutant and non-mutant P_1 fish in 2017		
Treatment or Mutants	N eggs	N embryo hatched	Hatch rate (%)	N fingerlings survived	Survival rate (%)	N fish	N fish survived	Survival rate (%)	N fish	N fish survived	Survival rate (%)
	200	47	23.5	32	68.1	10	3	30.0	4	3	75.0
Controls or Non-mutants	200	96	48	79	82.3	23	14	60.9	40	34	85.0

Notes:
Hatch rate was significantly different between treatment group and control group for P_1 embryo (Fisher's exact test, P<0.001).
Survival rates were not significantly different between treatment group and control group for P_1 6-months fingerlings (Fisher's exact test, P=0.086).
Survival rates were not significantly different between mutants and non-mutants for 3-year-old P_1 and 4-year-old P_1 (Fisher's exact test, P=0.141, P=0.514).

Table 12. Survival rate, mutation rate and mean body weight of 3 families of 1-year-old F_1 offspring spawned in 2017 of channel catfish (*Ictalurus punctatus*).

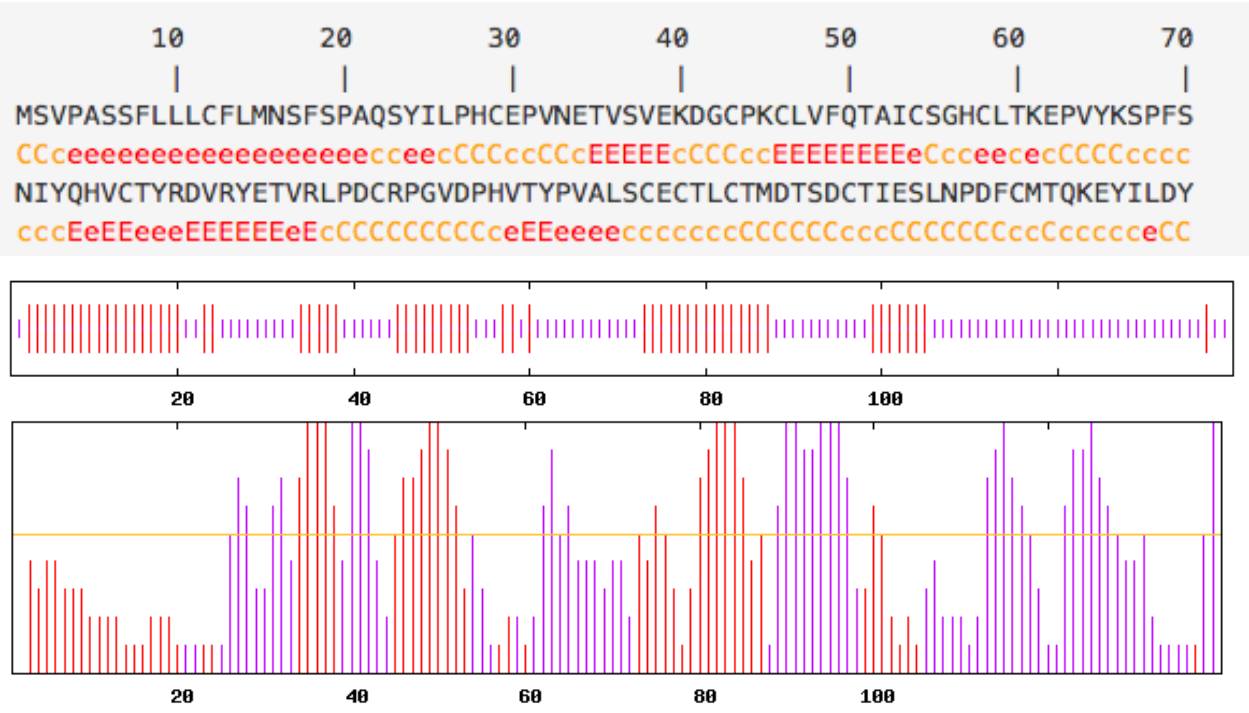
Different Families of F_1 offspring	Survival rate calculation			Mutation analysis			Mean body weight (g) ± SEM of mutant fish[a]	Mean body weight (g) ± SEM of non-mutant fish[a]
	N fish	N fish survived	Survival rate (%)	N fish sampled	N mutant fish	Mutation rate%		
2017 LH-1	300	276	92.0	26	13	50.0	5.30±0.508	6.77±0.742
2017 LH-2	300	262	87.3	26	0	0	/	/
2017 LH-3	200	173	86.5	26	13	50.0	10.18±0.523	8.92±0.751

Notes:
Survival rates were not significantly among different families of F_1 offspring (Fisher's exact test, P=0.078).
Body weight was not significantly different between mutant fish and non-mutant fish in each F_1 family, including 2017 LH-1 and 2017 LH-3 (Student's t test, P=0.119, P=0.184).

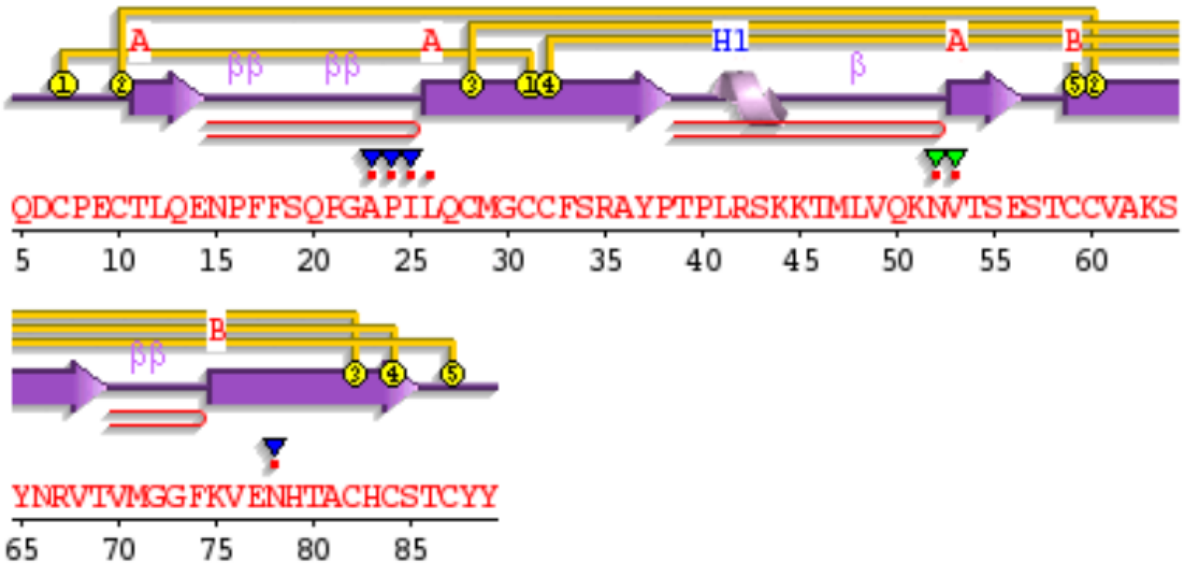
3.5 Sequence Structure Analysis

There are total 140 amino acids of LH protein. The alignment shows significant similarity with a cutoff E-value of 2e-29 between LH and Human chorionic gonadotropin. However, the alignment is located at the downstream of the cutting site stating from the 21st amino acid. The predicted secondary protein structure of LH contains extended strand (42.86%) and random coil (57.41%) (Fig. 20A). The predicted secondary protein structure of FSH protein using PDBsum includes 7 β-turns (Fig. 20B). Meanwhile, the tertiary structure of LH was identical to 1hcnB with C-score of -0.57, and the image was colored by rainbow from N to C terminus (Fig. 20C).

A


10 20 30 40 50 60 70
MSVPASSFLLLCFLMNSFSPAQSYILPHCEPVNETVSVEKDGCPKCLVFQTAICSGHCLTKEPVYKSPFS
NIYQHVCTYRDVRYETVRLPDCRPGVDPHVTYPVALSCECTLCTMDTSDCTIESLNPDFCMTQKEYILDY
20 40 60 80 100
20 40 60 80 100

B



Key:

Sec. struc: Helices labelled H1, H2, ... and strands by their sheets A, B, ...

Helix Strand

Motifs: β beta turn beta hairpin

Disulphides: disulphide bond

Residue contacts: to ligand

PDB SITE records: AC1 AC2

C

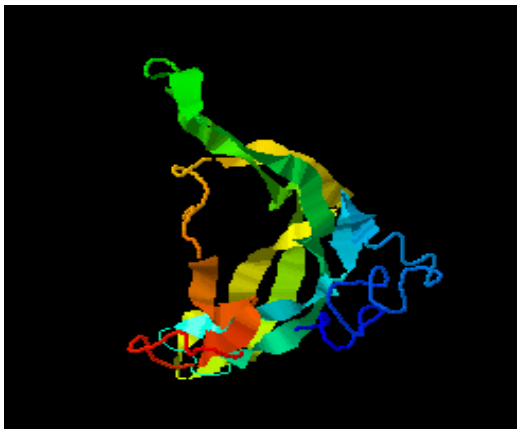


Figure 20. Prediction and analysis of luteinizing hormone (LH) protein structure. **(A)** Prediction and analysis of secondary structure of LH protein using Pole Bioinformatique Lyonnais (PBIL) server, which was categorized in extended strand (42.86%) and random coil (57.41%) Hh: Alpha helix; Ee: Extended strand; Cc: Random coil. **(B)** Secondary structure of LH protein was predicted using PDBsum Generate, which has 7 β-turns. **(C)** Three-dimensional structure of LH was predicted using I-TASSER server, which is identical to 1hcnB with C-score of -0.57, and the image was colored by rainbow from N to C terminus.

4 Discussion

In this study, indels were detected at around 60 bp upstream of the expected site of LH gene. In the P_1 generation, the mutagenesis attained 38.9%. Sequencing results showed that a variety of LH mutations were induced, including insertions, deletions, substitutions, and a complex of multiple types. These mutations were not located at the ZFN-targeted position, but all were located approximately 60 bp upstream of the target site. However, they were still located in the ORF of the channel catfish LH gene. None of the female mutants could spawn without hormone therapy for three consecutive years after sexual maturation. Mutant males had lower spawning rate than controls with a very low egg hatch rate of 1%. LHRHa, carp pituitary extract and carp LH didn't work well, while HCG hormone injection restored the fertility of mutant P_1 fish. No pleiotropic effects in terms of body weight and survival rate were detected from mutants.

Four LH mutant channel catfish did not spawn at 2 years of age, in contrast to 3 of 4 pairs of control catfish that successfully spawned under the same conditions. In the following year, 11 pairs of LH mutant channel catfish did not show courtship behavior and did not spawn despite, having excellent gravidity and other secondary sexual characteristics, whereas 11 out of 15 control pairs spawned fertile egg masses. Eleven pairs of 3-year-old fish did not spawn after hormone therapy with CPE. None of 6 4-year-old LH mutant females could spawn when paired

with wild type males. One of 4 4-year-old LH mutant male fertilized the eggs with low hatch rate when paired with wild type females. It appears that the desired loss of function, sterilization, was successfully achieved based on these results or reproductive ability greatly impaired. Similar results obtained in zebrafish (*Danio rerio*). LH-deficient zebrafish (*lhb*−/−) showed normal gonadal growth of both females and males. The females failed to spawn and were therefore infertile, while the males were fertile by their successful spawning with wild type females to produce normal offspring (Zhang et al. 2015).

Initial attempts to restore fertility failed for LH mutants, providing more evidence of successful sterility. Repeated attempts to spawn LH mutants with LHRHa and carp LH failed. Successful spawning and hatching was obtained for LH mutants by injection with 1,600 IU human chorionic gonadotropin (HCG). HCG is cross reactive with both LH and FSH receptors (Siris et al. 1978). HCG is a preferred therapy over LH in human medicine as HCG is very stable, whereas, LH has a very short half-life. Additionally, HCG is already an FDA approved spawning aid for fish.

Hypothetically, the mutations of LH gene could block the function of the gene and lead to sterilization of female fish. It is becoming increasingly evident that the differences in steroidogenic potency between teleost FSH and LH are related to the state of gonadal development. Whereas FSH and LH have similar steroidogenic potency during early phases of gametogenesis (Planas et al. 1993, Planas et al. 1995, Planas et al. 1997, Suzuki et al. 1988, Swanson et al. 1989, Van et al. 1922), LH is generally more potent than FSH during late stages of gametogenesis (Planas et al. 1995, Planas et al. 1997, Suzuki et al. 1988). Simultaneous presence of ovarian LH and FSH levels are needed during the reproductive cycle due to the presence of oocytes that are at different stages of maturation (Ko et al. 2007).

The function of LH gene for male fish remained obscure. LH receptors varies significantly among different species. The knockout of LH gene should restrain the synthesis and release of pituitary gonadotropin, followed by the repression of the secretion of steroid hormones, including estradiol and testosterone. However, despite the numerous reports on LH in teleosts in the past 20 years, we are still far from understanding the functions of LH in controlling fish reproduction.

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IV Generation of Gonadotropin Releasing Hormone and Luteinizing Hormone Gene-Edited Channel Catfish (*Ictalurus punctatus*) via Zygote Injection of CRISPR/Cas9 System

Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 is a new gene editing system with high targeting efficiency and low cell toxicity. The catfish type gonadotropin releasing hormone (cfGnRH) gene and luteinizing hormone gene are important because their roles in regulation of reproduction in channel catfish (*Ictalurus punctatus*). In this study, CRISPR/Cas9 was utilized to successfully target cfGnRH and LH genes respectively to sterilize channel catfish through microinjection. CRISPR/Cas9 induced 50% of mutagenesis in the target protein-encoding sites of cfGnRH and LH separately. No obvious effects on body weight were observed after the knockout of reproductive genes in the P_1 mutants. CRISPR-Cas9 nucleases enabled higher efficient genome editing than zinc finger nucleases (ZFNs), similar efficient genome editing than transcription activator-like effector nucleases (TALENs), however with low hatchability through microinjection.

1 Introduction

The functional characterization of the annotated genetic elements in normal biological processes is a major goal of fish genome project. Genome-scale loss-of-function screens have provided a wealth of information in diverse model systems (Shalem et al. 2014). RNA

interference (RNAi) is the predominant method for genome-wide loss-of-function screening in many species, including human (Berns et al. 2004, Boutros et al. 2004). Different strategies such as double-stranded RNA (dsRNA), short hairpin RNA (shRNA), miRNA, and small interfering RNA (siRNA) can be applied to achieve the goal (Saurabh et al. 2014). When exogenous RNA, which is synthesized with a sequence complementary to a gene of interest, is expressed in vivo, and the RNAi pathway activated, the target gene is silenced (Daneholt 2007). However, its utility is limited by the inherent incompleteness of protein depletion by RNAi and confounding off-target effects (Jackson et al. 2006, Echeverri et al. 2006).

The field of biology is now experiencing a transformative phase with the advent of facile genome engineering in animals and plants using RNA-programmable clustered regularly interspaced short palindromic repeats (CRISPR) with high efficiency and simplicity (Doudna and Charpentier 2014). CRISPR/CRISPR-associated (Cas) system is a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic elements so that protects bacteria and archaea from invading viruses and plasmids (Bhaya et al. 2011). CRISPR-Cas systems fall into 2 classes and 6 types based on locus organization and conservation (Makarova et al. 2006). Class 1 systems use a complex of multiple Cas proteins to degrade foreign nucleic acids. Class 2 systems use a single large Cas protein for the same purpose. Class 1 is divided into types I, III, and IV; class 2 is divided into types II, V, and VI (Barrangou and Marraffini 2014).

Type II systems are defined by the popular Cas9 endonuclease, containing Cas9, crRNA and trans-activating crRNA (tracrRNA) (Garneau et al. 2010), guides that direct the RuvC and HNH nickase domains to generate precise blunt DNA breaks in target DNA sequences flanked by a 3′ Protospacer adjacent motif (PAM) (Deltcheva 2011). Cas9 is an RNA-

guided DNA endonuclease enzyme associated with the CRISPR adaptive immunity system in *Streptococcus pyogenes*, among other bacteria. Each crRNA unit contains a 20-nt guide sequence and a partial direct repeat, where the former direct Cas9 to a 20bp DNA target. The crRNA and tracrRNA can be fused together to create a chimeric, single-guide RNA (sgRNA) (Ran et al. 2013). Guide RNA (gRNA) design is based on the crRNAs and tracrRNAs which naturally exist in nature. Cas9 can thus be re-directed toward almost any target of interest in the genome. The RuvC Nuclease initiates cleavage of the DNA strand not complementary to the gRNA. The HNH nuclease domain of Cas9 cleaves the DNA strand complementary to the gRNA. Protospacer adjacent motif (PAM) is a 2-6 base pair DNA sequence immediately following the DNA sequence targeted by the Cas9 nuclease in the CRISPR bacterial adaptive immune system (Sarah et al. 2013).

Cas9 promotes genome editing by stimulating a double-strand break (DSB) at target locus. Upon cleavage by Cas9, the target locus typically undergoes the Non-homologous end joining (NHEJ) and Homology-Directed Repair (HDR). In the absence of a repair template, DSBs are religated by the NHEJ process, which leaves scars in the form of insertion or deletion mutations, referred to as indels, which can lead to frameshift and premature stop codons.

Cas9 offers several potential advantages, including the higher targeting efficiency, the ease of customization, and the ability to facilitate multiplex genome editing. Cas9 can be easily retargeted to new DNA sequences by simply changing the 20-nt guide sequence. The Cas9 derived from *Streptococcus pyogenes* is known to make a DSB between the 17th and 18th bases in the targeted sequences (Jinek et al., 2012), whereas Transcription activator-like effector nucleases (TALENs) cleave nonspecifically in the 12-24bp linker between the pair of binding

sites. In addition, CRISPR/Cas9 system can be applied to target multiple genomic loci simultaneously by co-delivering a combination of sgRNAs (Jao et al., 2013).

CRISPR can be adopted for genome-scale screening by combining Cas9 with pooled-gRNA libraries and next-generation sequencing (NGS) (Korkmaz 2016). CRISPR/Cas9 library screening enables the identification of critical components in kinds of biological processes. CRISPR screen has recently been used to analyze synthetic lethal genes (Han 2017), HIV host dependency factors (Park 2017), genetic vulnerabilities in cancer (Manguso 2017, Song 2017), novel targets of diseases (Yamauchi 2018, Brunen 2018, Zhang 2018) and resistance phenotypes (Kurata 2016). Moreover, the identification of non-coding sequences and characterized enhancer elements and regulatory sequences belong to a novel application scope of CRISPR screens.

Cas9 Endonuclease Dead (dCas9), is a mutant form of Cas9 whose endonuclease activity is removed through point mutations in its endonuclease domains. Although dCas9 lacks endonuclease activity, it is still capable of binding to its gRNA and the DNA strand that is being targeted. This protein has been applied as a DNA-binding platform by using a variety of effector domains for transcription modulation and epigenetic editing. For example, dCas9 can knock out target gene transcription by fusing a repressive effector domain such as the Krüppel associated box (KRAB) domain. KRAB repression is mediated by repressive histone modifications (Gilbert 2013, Chen 2013).

The CRISPR/Cas9 system has been applied for cell therapies, mainly involving immune cell therapy and stem cell therapy (Chen 2013). Treatment using *ex vivo* gene-editing T cells from patients with cancer or autoimmune diseases have been derived (Ren 2017, Safari 2018). Apart from T cells, *ex vivo* editing of induced pluripotent stem cells (iPSCs) and hematopoietic stem cells (HSCs) derived from the patient's somatic cells also offer the opportunity for

investigating the pathophysiological mechanisms of hereditary diseases. With this technology, researchers have made advances in the treatment of, hemophilia B, Alzheimer disease, oculopathy and other genetic diseases (He 2017, Arber 2017, Ramsden 2017).

In recent years, CRISPR/Cas9 system has been applied for editing human embryos. Ma et al. (2017) described the correction of the heterozygous MYBPC3 mutation, which causes hypertrophic cardiomyopathy, in human preimplantation embryos with precise CRISPR–Cas9-based targeting accuracy and high homology-directed repair efficiency. Some researchers used the CRISPR/Cas9 system to correct HBB (A>G) mutation, which is one of the three most common mutations in China and Southeast Asia patients with β-thalassemia in the human embryonic genome (Liang 2017, Zhou 2017). These examples implied the tremendous potential of correcting homozygous and compound heterozygous mutations by base editing in human embryos.

In the field of fish research, CRISPR/Cas9 mediated gene knock-out was first performed in zebrafish by Hwang et al. (2013) with somatic mutagenesis rates ranging from 24 to 59% in 10 loci. Jao et al. (2013) were able to achieve 75-99% mutagenesis with most cells being bi-allelic, essentially achieving homozygous knockouts in the initial generation of zebrafish, which could be used for phenotypic evaluation, using the CRISPR/Cas9 system. A number of successful studies have exploited the CRISPR/Cas9-mediated zebrafish to test the causal role of specific genetic perturbations in a ‘genotype-to-phenotype’ approach (Hwang et al. 2013, Jao et al. 2013, Shah et al. 2015, Varshney et al. 2015). For example, Perles et al. employed the CRISPR/Cas9-mediated zebrafish to investigate the effect of MMP21 knock-out (Perles et al. 2015). MMP21 gene was suggested to be associated with human heterotaxy. The CRISPR/Cas9 system has been used to induce mutations in many animals including zebrafish (Chang et al.

2013, Hwang et al. 2013, Jao et al. 2013), medaka (Oryzias latipes) (Fang et al. 2018), common carp (Zhong et al. 2016), tilapia (Li et al. 2014), and Atlantic salmon (Edvardsen et al. 2014). In recent years, CRISPR/Cas9 technology has also been successfully used in channel catfish, which is the most important freshwater aquaculture species in the USA, for a variety of genes (Khalil et al. 2017, Qin 2015, Elaswad 2016, Qin et al. 2016, Elaswad and Dunham 2017, Elaswad et al. 2017, Khalil et al. 2017, Qin 2017, Dunham et al. 2018). Rolf et al. (2014) used the CRISPR/Cas9 system to target two genes involved in pigmentation, tyrosinase (tyr) and solute carrier family 45, member 2 (slc45a2) in Atlantic salmon, which shows for the first-time successful use of the CRISPR/Cas9 technology in a marine cold water species.

In this study, CRISPR/Cas9 technology was utilized for the targeted gene disruption of cfGnRH gene and LH gene to sterilize channel catfish. Microinjection approach was performed and evaluated. The procedures developed in this study would also greatly facilitate knockout studies for functional genomic experimentation.

2 Materials and Methods

2.1 Preparation of sgRNA and CRISPR/Cas9 System and experiments design

Guide RNAs used in the study are listed in Table 1. Three gRNAs for cfGnRH (GenBank Accession No. XM_017468372.1) and 2 gRNAs for LH (GenBank Accession No. AF112192.1) genes were designed using CRISPRscan gRNA design tool (Moreno-Mateos et al. 2015). Guide RNAs were prepared according to Khalil et al. (2017) using Platinum™ Taq DNA Polymerase (Invitrogen). Cas9 protein was purchased from PNA BIO Inc. (Newbury Park, CA). For cfGnRH gene, equal amounts of the 3 gRNAs were mixed with Cas9 protein before injection. For LH, equal amounts of the 2 gRNAs were mixed with Cas9 protein before injection (Table 13). Phenol

red was added to color the sgRNA/Cas9 solutions by mixing sgRNA, Cas9 protein and phenol red in a 1:1:1 ratio. The concentrations of gRNAs and Cas9 proteins were adjusted to 150-200 ng μl^{-1}, so that the injection of 50nL will deliver approximately the amount needed for each embryo. The mixtures were incubated for 10 minutes on ice before loading into the microinjection needle.

The experiment included 2 treatments and 2 control groups for each of cfGnRH and LH genes. Fifty nL of gRNA/Cas9 protein for each gene were injected, while the first control group was injected with the same volume of buffer without gRNA or Cas9 protein (injected control, iCTRL). The second control was not microinjected (nCTRL). The treatment of cfGnRH gene had 3 replicates, while the treatment of LH gene had 4 replicates. Each of the nCTRL and iCTRL groups had 1 replicate. All embryos in treatment and control groups for each gene were full-siblings, exposed to the same handling stress and reared in the same environmental conditions.

Table 13. The sequences of small guide RNAs and the universal (common) primer used to target exon 2 of catfish type gonadotropin releasing hormone (cfGnRH) gene and subunit of luteinizing hormone (LH) gene in channel catfish (*Ictalurus punctatus*).

Guide RNA ID	Oligo sequence (5'-3')	Locus on strand	CRISPRscan score %
cfGnRH-1	aatacgactcactataGGGGATGGTGGTGTGTGTTGgttttagagctagaa	+	82
cfGnRH-2	taatacgactcactataGGGAGCACTCTGGTGGATGGgttttagagctagaa	+	74
cfGnRH-3	taatacgactcactataGGCAGCGTTGCAGGAGGTAAgttttagagctagaa	+	73
LH-1	taatacgactcactataGGAGTGCCCGCTGCAGATGGgttttagagctagaa	-	82
LH-2	taatacgactcactataGGCTGACTGTCGGCCCGGTGgttttagagctagaa	+	76
Universal primer	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGC TATTTCTAGCTCTAAAAC		

2.2 Experimental Brood Stock and Gametes

One sexually mature female of Kansas Random strain of channel catfish (*Ictalurus punctatus*) was selected and implanted with luteinizing hormone releasing hormone analog (LHRHa) (Reproboost® Implants, Center of Marine Biotechnology, Columbus Center, Maryland, USA) with a dose of 90 μg kg^{-1}. It showed outstanding secondary sexual characteristics. Eggs of the anesthetized ovulating females were hand stripped into dried stainless steel pie pans greased with vegetable shortening. All eggs were collected for each experimental group and control group. One Kansas Random strain male (Dunham and Smitherman, 1983) was euthanized. Mixed testes were placed into a mesh strainer and macerated manually into saline (0.9% NaCl) to collect sperm. Eggs were fertilized in batches of 200-300, and embryo microinjection was initiated 15 min after fertilization and continued for 60-90 min (embryos were still in the one-cell stage).

2.3 Microinjection, Embryo Incubation and Hatching

The fertilized eggs were injected according to the procedures of zygote injection developed and modified recently in our laboratory (Elaswad 2016). Briefly, a 1.0 mm OD borosilicate glass capillary was pulled into two needles with a vertical needle puller. A very thin layer of vegetable shortening was applied to a 150 mm clean petri dish. Fifty to 100 eggs were transferred from the fertilization pan to the petri dish in a single layer and covered with Holtfreter's solution (59 mM NaCl, 0.67 mM KCl, 2.4 mM $NaHCO_3$, 0.76 mM $CaCl_2$, 1.67 mM $MgSO_4$) (Bart et al. 1996). Using a microinjection system from Applied Scientific Instrumentation (Eugene, OR), 50 nanoliters of the mixture were directly injected into the yolk.

One cell embryos were injected through 15 - 90 minutes post fertilization and just before the beginning of the first cell division (Tucker and Robinson 1990). The injected control embryos were injected with the solution devoid of Cas9/gRNA mixture.

Immediately after microinjection, embryos were incubated in Holtfreter's solution (59 mM NaCl, 0.67 mM KCl, 2.4 mM NaHCO3, 0.76 mM CaCl2, 1.67 mM MgSO4) with 100 ppm doxycycline. Each replicate of a treatment was incubated in a plastic tub containing 7 liters of Holtfreter's solution and continuous aeration. Incubation temperature ranged from 26 to 28°C while dissolved oxygen levels were kept above 5 ppm using air diffusers. All tubs for each gene were held in a rectangular tank containing pond water to reduce the fluctuations in temperatures and ensure all the experimental units had the same temperature.

Dead embryos were removed and recorded daily. Embryos were not handled in the first 24 hours of incubation. At the time of dead embryo removal, Holtfreter's solution was replaced and the incubation tubs were cleaned. The temperature of Holtfreter's solution was monitored and adjusted to the same degree as the old solution to minimize the adverse effects of temperature fluctuation on embryos. The hatching temperature was 28°C.

2.4 Fish Culture

After hatch, the fry were reared in Holtfreter's solution without doxycycline until 10 days post fertilization (dpf). They consumed their yolk sac and began to swim up (at 9-10 dpf). They were fed *artemia nauplii* (Pentair Aquatic Eco-systems, USA) three times per day to ensure continuous feed supply. Starting at 10 dpf, complete water exchange was done every third day with one-third of Holtfreter's solution replaced with pond water each time until two-thirds of the

Holtfreter's solution was replaced with pond water. Water quality parameters were monitored daily.

Starting at 30 dpf, they were stocked into a recirculating system with densities of 20 fish per 60L aquaria of each family. At the beginning, fry were fed Purina® AquaMax® Fry Powder (Purina, St. Louis, MO) three times a day. As fry grew they were then fed Purina® AquaMax® Fry Starter 100. After further growth fry were fed Purina® AquaMax® Fry Starter 200 and 300 three times a day.

2.5 Sample Collection and High Fidelity PCR

Embryos were cultured for around two weeks in the tub and then transferred into a recirculating system. After 6 months, the genomic DNA was extracted from the pelvic fin sample. Samples were digested with cell lysis buffer and proteinase K following the protocols by Cheng et al. (2014). DNA agarose gel electrophoresis and spectrophotometry were then used to test the quantity and quality of the genomic DNA. Then Roche Expand High FidelityPlus PCR System (Roche, Indianapolis, IN) was used to amplify the cfGnRH gene and LH gene on these DNA samples. The following primers were utilized for amplifying cfGnRH gene: forward sequence 5'-ATGGATGCTGTCTTTGTTTTCC-3'; reverse sequence 5'-CCACACGAAATAAAGGCAAAG-3'. The following primers were utilized for amplifying LH gene: forward sequence 5'- AGGATGTCAGTGCCAGCTTC -3'; reverse sequence 5'-CTTGGAGTAAATGGACTCGTTG -3' (Table 14). The amplification procedure was: initial denaturation for 2 min at 94°C, followed by 29 cycles of 94°C for 30s, 60°C for 30s and 72°C for 40s, and the final elongation for 10min at 72°C.

Table 14. Primer sequences used for the amplification of catfish type gonadotropin releasing hormone (cfGnRH) and luteinizing hormone (LH) genes in channel catfish (*Ictalurus punctatus*).

Targeting Site		Sequence
cfGnRH	Forward	5'-ATGGATGCTGTCTTTGTTTTCC-3'
	Reverse	5'-CCACACGAAATAAAGGCAAAG-3'
LH	Forward	5'- AGGATGTCAGTGCCAGCTTC -3'
	Reverse	5'- CTTGGAGTAAATGGACTCGTTG -3'

2.6 TA Clone and Sequencing

TOPO® TA Cloning® kit was used to set up a 6μL TOPO cloning system, which included 4μL amplified DNA product, 1μL salt solution and 1μL TOPO vector. The system was mixed and incubated under room temperature (22-23°C) for 5min, followed by competent cell transfection. First, 2μL of the cloning system was added into one vial of One Shot®TOP10F'competent cells and put on the ice for 30min. Then 42°C heat shock was implemented using PCR machine for 30s. After that, the tube was placed on ice immediately. The next, 250μL LB (Tryptone 10g L^{-1}, Yeast extract 5 g L^{-1}, NaCl 10 g L^{-1}, Agarose 10g) broth was mixed together in a 1.5ml tube and placed in the 37°C shaker and shaken at 200rpm for 1 hour. Next, 50μL, 80μL, and 100μL mixtures were spread on the LB (Tryptone 10 g L^{-1}, Yeast extract 5 g L^{-1}, NaC l10 g L^{-1}, Agarose 10g) medium and placed in the 37°C incubator overnight. Ten monoclonal colonies were picked from each plate, mixed with 400ml LB (Tryptone 10 g L^{-1}, Yeast extract 5 g L^{-1}, NaCl 10 g L^{-1}, Agarose10g) broth and placed in the 37°C incubator overnight. The amplified bacterial solution was dispensed into a 96-wells plate and sent to the Eurofins Genomics Company (Louisville, KY, USA) for sequencing. Finally, sequencing results were analyzed using T-Coffee tool (Notredame et al. 2000).

2.7 Pleiotropic Effects

Hatch rate was recorded for all embryos. Survival rate was measured at 1 month after hatch. Body weight for 6-month-old treatment, iCTRL and nCTRL groups of fingerling were recorded and measured for evaluating the pleiotropic effects.

2.8 Statistical Analysis

Statistical analysis of hatch rate and body weight were performed with R studio software (RStudio Inc, Boston, MA). Student's *t*-tests were used to compare body weight between treatment group and control group. Fisher's exact tests were performed to compare hatch rate and survival rate between treatment group and control group. Comparisons of statistical significance was set at $P<0.05$, and all data were presented as the mean ± standard error (SEM).

3 Results

3.1 Analysis of Mutation Efficiency of cfGnRH gene

Sequence and alignment analyses confirmed that cfGnRH gene was successfully mutated in 5 of 10 6-month-old fingerling with a 50.0% mutation rate in channel catfish (*Ictalurus punctatus*). All the mutations occurred within the gRNA target cutting sites. Different types of mutation were found, including base deletion and substitution (Fig 21A and B).

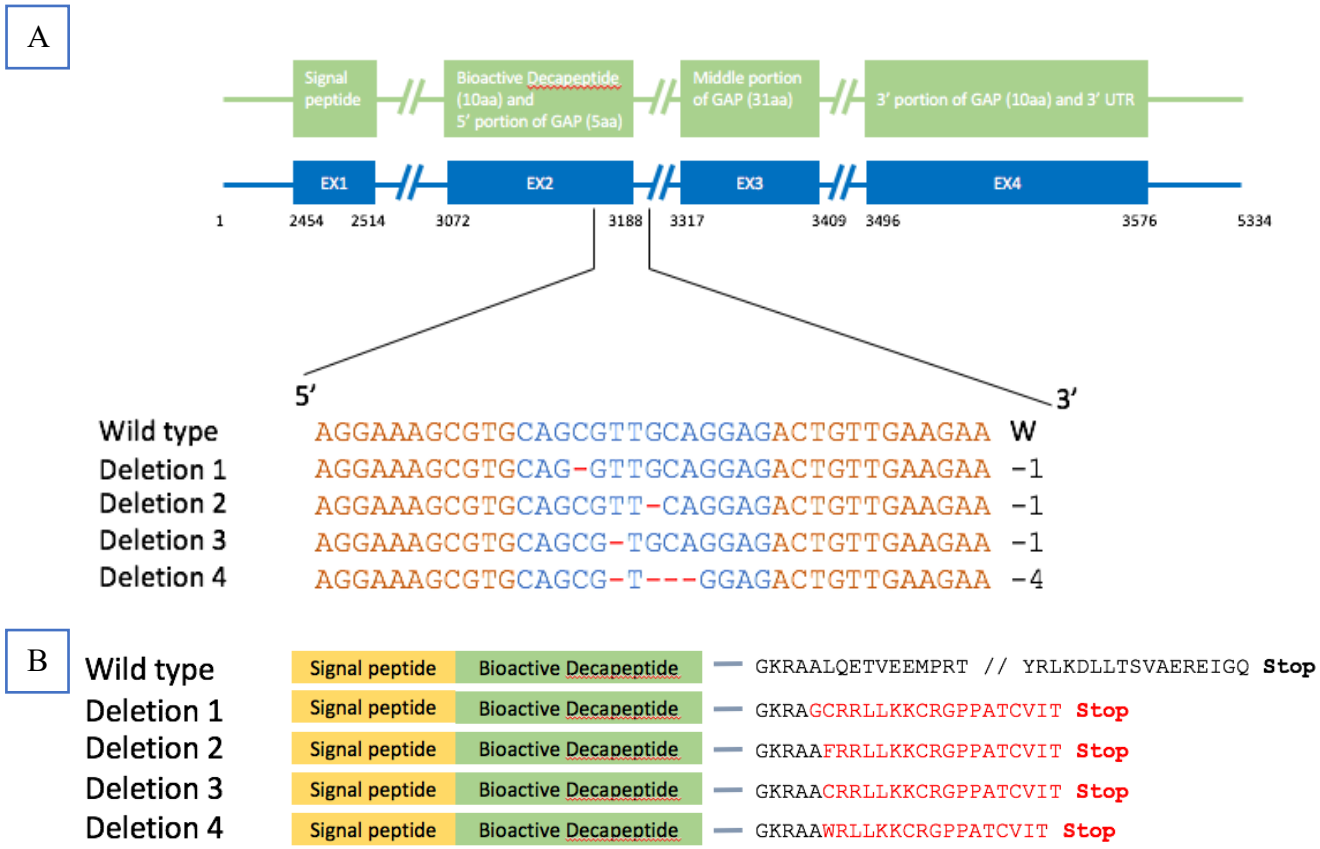

Figure 21. Sequences and predicted amino acid sequences of catfish type gonadotropin releasing hormone (cfGnRH) gene in channel catfish (*Ictalurus punctatus*). **(A)** The wild-type channel catfish cfGnRH gene sequences are shown on the top. Sequences in blue are the original sequences. Red dashes indicate the deletion of nucleotides. Numbers at the end of the sequences show the number of nucleotides deleted (-) in the edited cfGnRH gene. **(B)** Amino acid sequences following incomplete domain were due to fame-shift reading, resulting in a premature stop (red color) codon.

3.2 Analysis of Mutation Efficiency of LH gene

Sequence and alignment analyses confirmed that LH gene was successfully mutated in 5 of 10 6-month-old fingerling with a 50.0% mutation rate in channel catfish (*Ictalurus punctatus*).

All the mutations occurred within the LH target cutting sites. Different types of mutation were found, including base deletion, insertion and substitution. (Fig. 22A and B).

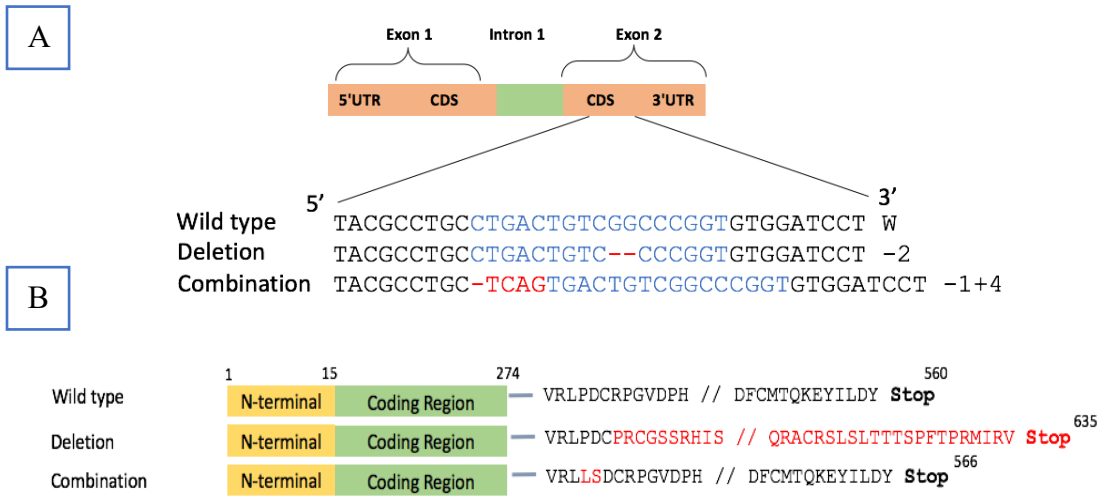

Figure 22. Nucleotide sequences and predicted amino acid sequences of luteinizing hormone (LH) gene in channel catfish (*Ictalurus punctatus*). (A) The wild-type channel catfish LH gene sequences are shown on the top. Sequences in blue are the original sequences. Red dashes and letters indicate the deletion and insertion of nucleotides. Numbers at the end of the sequences show the number of nucleotides deleted (-) or inserted (+) in the edited LH gene. (B) Red letters of amino acid sequences were due to fame-shift reading.

3.3 Pleiotropic Effects

Embryos hatch rates ranged from 0-42.4% for cfGnRH groups and 4-23.1% for LH groups. Survival rates were both 100.0% for cfGnRH groups and ranged from 41.7-100.0% for LH groups. Injected control had hatch rate of 11.8% and survival rate of 81.8%, while non-injected control had hatch rate of 75.8% and survival rate of 92.0% (Table 15).

Hatch rates were different among 3 cfGnRH treatment repeats and 4 LH treatment repeats respectively (P<0.05). Hatch rates were not different for the following groups: cfGnRH-

2, LH-1, LH-4, and iCTRL (P>0.05). However, significant difference was found between all the injected groups and nCTRL (P<0.05) (Table 15).

Table 15. The survival and hatchability of embryos of the channel catfish (*Ictalurus punctatus*) microinjected at one-cell stage with sgRNAs/Cas9 protein targeting the catfish type gonadotropin releasing hormone (cfGnRH) and luteinizing hormone (LH) genes individually and their full sibling injected control (iCTRL) and non-injected control (nCTRL). Hatch rate % is the number of total hatched embryos in each treatment divided by the total number of embryos in the same treatment and multiplied by 100. Survival rate % is the number of live embryos after hatch in each treatment divided by the total number of embryos in the same treatment and multiplied by 100. Pairwise comparisons of hatch rate and survival rate were performed using Fisher's exact test with R-studio software. Hatch rate and survival rate followed by different superscript letters are significantly different (P<0.05).

Treatment	N eggs	N embryo hatched	Hatch rate (%)	N fry	N fry survived	Survival rate (%)
cfGnRH-1	60	0	0^{a}	/	/	/
cfGnRH-2	95	14	14.7^{ab}	14	14	100.0^{a}
cfGnRH-3	33	14	42.4^{c}	14	14	100.0^{a}
LH-1	92	12	13.0^{ab}	12	5	41.7^{b}
LH-2	75	3	4.0^{a}	3	2	66.7^{a}
LH-3	91	21	23.1^{b}	21	15	71.4^{a}
LH-4	89	16	18.0^{ab}	16	16	100.0^{a}
iCTRL	93	11	11.8^{ab}	11	9	81.8^{a}
nCTRL	33	25	75.8^{d}	25	23	92.0^{a}

cfGnRH and LH genes were successfully mutated in 5 of 10 6-month-old fingerling with a 50.0% mutation rate in channel catfish (*Ictalurus punctatus*) respectively. No significant differences were found between mutants and non-mutants for both cfGnRH group and LH group (P>0.05), indicated no obvious pleiotropic effect on body weight was found (Table 16).

Table 16. Mutation rate and mean body weight of channel catfish (*Ictalurus punctatus*) fingerlings microinjected at one-cell stage with single guide RNAs (sgRNAs)/Cas9 protein targeting the catfish type gonadotropin releasing hormone (cfGnRH) and luteinizing hormone (LH) genes individually. Mutation rates are calculated based on sequencing results of 6-month-old fingerlings. All body weight data are presented as the mean ± standard error (SEM).

Genotype	Mutation analysis			Mean body weight (g) ± SEM of mutant fish	Mean body weight (g) ± SEM of non-mutant fish
	N fish sampled	Mutation rate%	N mutant fish		
cfGnRH	10	50.0	5	12.80±1.05	10.90±0.43
LH	10	50.0	5	33.20±2.88	29.70±2.23

Notes:
Mutation rates were not significantly different between cfGnRH treatment group and LH treatment group for 6-months fingerlings (Fisher's exact test, P=1.000).
Body weights were not significantly different between mutants and non-mutants for (Student's t test, P=0.309, P=0.322).

4 Discussion

The sgRNA(s) and Cas9 protein were directly co-injected into one-cell embryos, and the targeted mutations were detected in cfGnRH gene. Different types of mutation were found, including base deletions and substitutions with the total number of base changes ranging from 1-5. Designed sgRNA of cfGnRH-3 worked effectively at the target site on cfGnRH gene with 50% mutagenesis. Embryos hatch rates were ranged from 0-42.4%, which is different with nCTRL of 75.8%. Fry survival rate were both 100.0%, which is not different with nCTRL. No significant differences were found between mutants and non-mutants. The targeted mutations were also detected of LH gene. Different types of mutations were found, including base deletions, insertions and substitutions with the total number of base changes ranging from 1-5. Designed sgRNA of LH-2 worked effectively at the target site on cfGnRH gene with 50% mutagenesis. Embryos hatch rates ranged from 4-23.1%, which is different with nCTRL of 75.8%. Fry survival rate ranged from 41.7-100.0%, which is not different from nCTRL. No significant differences in terms of body weight were found between mutants and non-mutants.

Microinjected embryos had low hatchability, when compared to their nCTRL siblings. Injected siblings had low hatchability also, which indicated the low hatchability may be due to the microinjection procedures since all embryos were full-siblings, exposed to the same handling stress, and reared using the same environmental conditions. There is no information on cfGnRH and LH pleiotropic effects and if knockout of the two genes would affect survival or body weight. In the current study, survival rates after hatching of microinjected fry were not different

with nCTRL. Body weights were also not different between mutants and non-mutants for neither cfGnRH group nor LH group. However, successful knockout of those genes has been achieved, so the possibility of the knockout affecting the survival of channel catfish embryos cannot be eliminated without further investigation. Pleiotropic effects for gene knockout were previously reported in myostatin-deficient medaka (*Oryzias latipes*) (Yeh et al. 2017). The possibility of the effects of off-target mutations on fingerling or fish survival and body weight exists.

CRISPR-Cas9 nucleases enable highly efficient genome editing in a wide variety of organisms, including nematode (*Caenorhabditis elegans*) (Friedland et al. 2013, Ward 2015), fruit fly (*Drosophila*) (Gratz et al. 2013), frog (*Xenopus tropicalis*) (Nakayama et al. 2013), zebrafish (*Danio rerio*) (Chang et al. 2013, Hwang et al. 2013), channel catfish (Khalil et al. 2017), mice (Wang et al. 2013, Yang et al. 2013) and human cells (Shalem et al. 2014, Wang et al. 2014). High efficiency and low cell toxicity have been reported, but unwanted mutations at off-target sites that resemble the on-target sequence can also be caused (Cradick et al. 2013, Fu et al. 2013, Hsu et al. 2013, Pattanayak et al. 2013, Tsai et al. 2015). Off-target effects may be affected by the design of gRNA, the method of off-target mutation detection or the genes being targeted. Cho et al. (2013) showed it's important to choose unique target sequences and optimize guide RNA and Cas9 to avoid or reduce RNA-guided endonucleases-induced off-target effects.

In the previous studies, TALENs plasmids targeting the cfGnRH and FSH genes were delivered into fertilized eggs with double electroporation to sterilize channel catfish respectively. Targeted mutagenesis was found in 52.9% P_1 fish for cfGnRH gene, and 63.2% P_1 fish for FSH gene. Different types of mutation were found, including base deletion, substitution, and insertion with the total number of base changes ranging from 1-5. ZFNs plasmids were delivered targeting the LH gene into fertilized eggs with double electroporation to sterilize channel catfish. Targeted

LH gene mutagenesis was found in 38.9% P_1 fish. Different types of mutation were found, including base deletion, substitution, and insertion with the total number of base changes ranging from 1-2. Designed sgRNA of cfGnRH and LH genes worked effectively at the target site with 50% mutagenesis respectively. Base deletion, insertion and substitution were found with the total number of base changes ranging from 1-5. CRISPR-Cas9 nucleases enabled higher efficient genome editing than ZFNs, similar efficient genome editing than TALENs, same types of mutagenesis as ZFNs and TALENs for single gene knock-out, but with low hatchability through microinjection. However, for multiple genes knock-out, CRISPR/Cas9 system could be used to achieve large genomic deletions resulting from the injection of the multiple sgRNAs in combination (Khalil et al. 2017), not only in one gene, but also in different chromosomal loci by delivering multiple pairs of sgRNAs together, with Cas9 nuclease targeting different genomic sites simultaneously with much convenience, efficiency and lower costs compared with ZFNs and TALENs plasmids (Cong et al. 2013, Jao et al. 2013, Wang et al. 2013).

Electroporation and microinjection were both utilized to guarantee mutant transgenesis in channel catfish. (Qin 2015, Qin et al. 2016). Whether to choose electroporation or microinjection could be decided by the aim of the study, and for large scale application, electroporation should be superior because of its simplicity and convenience. Further study is needed to evaluate the efficiency of other gRNAs, and the phenotypic traits and pleiotropic effects on adult mutants.

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V Conclusion

The utilization of genetically engineered catfish has great potential to provide greater sustainability and profitability for US catfish industry. Fish sterilization technology could minimize the environmental risk from escapees or released genetically engineered individuals to natural populations. To sterilize channel catfish, zinc finger nuclease (ZFN) was performed to knock out luteinizing hormone (LH) gene. Transcription activator-like effector nuclease (TALEN) was applied to induce mutagenesis of catfish type gonadotropin releasing hormone (cfGnRH) and follicle-stimulating hormone (FSH) genes respectively. Compared with plasmid electroporation, gRNA mixd with Cas9 were microinjected to knock out cfGnRH gene and LH gene respectively.

Targeted cfGnRH gene and FSH gene mutagenesis were found in 52.9% and 63.2% P_1 fish through TALEN technology respectively. For cfGnRH treatment, P_1 mutants had spawning rates of 20.0-50.0% when there was no hormone therapy with average egg hatch rates of 2.0%, which is lower than controls, except for one GnRH female that had a 66.0% hatch rate. Luteinizing hormone releasing hormone analog (LHRHa) hormone therapy resulted in good spawning and hatch rates for mutants, which were not significantly different from controls. For FSH treatment, P_1 mutants had spawning rates of 33.3-40.0% when there was no hormone therapy with average egg hatch rates of 0.75%, which is different with controls. Human chorionic gonadotropin hormone therapy resulted in spawning rates of 80% for female mutants and 88.9% for male mutants, and mean hatch rate of 35.0% for F_1 embryo, which were not significantly different from controls.

LH gene mutagenesis was found around 60 bp upstream of expected site in 38.9% P_1 fish through ZFN technology. Channel catfish females carrying the mutated LH gene were sterile, as confirmed by DNA sequencing and mating experiments. None of the female mutant fish could spawn without hormone therapy. P_1 mutant males had lower spawning rate than controls with egg hatch rates of 1%. HCG hormone therapy resulted in good spawning and hatch rates for mutants, which were not significantly different from controls.

Through CRISPR/Cas9 technology, 50% mutagenesis were attained on both cfGnRH treatment fish and LH trearment fish. No significant difference in terms of body weight were detected between mutants and non-mutants indicated no obvious pleiotropic effects. However, compared with electroporation, microninjection has much lower eggs hatch rate on P_1 generation. No difference in terms of mutagenesis rate were found in three different gene editing technology. ZFN plasmid electroporation induced mutations located around 60bp upstream of the proposed targeted site. TALEN and CRISPR/Cas9 induced mutatitons located at the expected cutting site.

Hormone therapy successfully restored the fertility of sterilized mutants. LHRHa was utilized for cfGnRH knocked out fish, HCG was applied for both FSH and LH knocked out fish. Generating sterile P_1 channel catfish through gene editing technology and then restoring their fertility to get brood stock appears promising.

By 2025, aquaculture will need to increase by 350% worldwide to cover the impending seafood shortage, and by 2030 an additional 29 million tons of fish will be needed for human consumption (Cressey 2009). Unfortunately, the United States is the leading global importer of fish and fishery products (NOAA 2016), and 91% of the seafood consumed by value is imported, resulting in a trade deficit, which is increasing every year and is now $11.2 billion annually. The

United States is not in a position to take advantage of the opportunity of the expanding global market of aquaculture-grown fish, including catfish (Dunham and Elaswad 2018).

Exploiting fish genetics can greatly contribute to production and efficiency. The growth hormone (GH) transgenesis accelerated the growth of channel catfish (Dunham et al 1992) as well as in many other species, including mice (*Mus musculus*) (Palmiter et al. 1982), Coho salmon (*Oncorhynchus kisutch*) (Devlin et al. 1999), Atlantic salmon (*Salmo salar*) (Du et al. 1992), rainbow trout (*Salmo gairdneri*) (Agellon et al. 1988), and common carp (*Cyprinus carpio*) (Chatakondi et al. 1995). β-actin-masou salmon desaturase transgenic channel catfish produced 25% more Ω-3 fatty acids compared to controls (Bugg 2017), and similar results have been found in common carp (Cheng et al. 2014) and zebrafish (Wang et al. 2014). Other traits such as disease resistance and resistance to abiotic factors have been improved via transgenesis in fish, including zebrafish (Peng et al. 2010) and channel catfish (Dunham et al. 1992). Gene editing has also altered traits such as growth in mice (Srisai et al. 2011), yellow catfish (*Pelteobagrus fulvidraco*) (Dong et al. 2011) and channel catfish (Khalil et al. 2017).

Because of public concern for environmental risk, transgenic and gene-edited fish are regulated by the Food and Drug Administration under the Federal Food, Drug, and Cosmetic Act and the Public Health Service Act (Dunham 2006, Nielsen 2003). For the commercial application of the genetic gains made through these two approaches, 100% reproductive confinement is necessary or should be the ultimate goal to prevent permanent environmental impact, negative or positive, of these genetically altered fish. Our initial results that gene editing of the cfGnRH locus has great potential to achieve the goal of reproductive or biological confinement, the procedure is relatively straightforward and normal spawning protocols utilizing LHRHa application can temporarily restore fertility as needed. Next, cfGnRH channel catfish

need to be generated that are also transgenic or edited for other genes to determine the feasibility of actual application of the technology. There is the possibility that gene editing of reproductive genes could decrease the benefit of growth hormone gene transfer or other genes such as was the case with triploid GH transgenic fish (Jhingan et al. 2003, Razak et al. 1999). Until evaluated, potential epistatic effects of mutated alleles with transgenes or other mutated loci are unknown and need to be examined.

Ultimately, gene editing of reproductive genes coupled with hormone therapy could be used in a variety of fish, including any domestic genotype, invasive fish or transgenic fish. These technologies would minimize impacts on the natural environment to protect genetic biodiversity and ecosystems and increase environmental friendliness of aquaculture and transgenic fish.

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