Genetic Technologies for Disease Resistance Research and Enhancement in Catfish

by

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Abstract

A microinjection protocol was developed and validated for CRISPR/Cas9 gene editing in channel catfish, *Ictalurus punctatus*. This protocol proved to be rapid, efficient and does not require elaborate equipment or a high level of technical skill. To demonstrate the efficiency of the protocol, two channel catfish immune-related genes were targeted, toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene. Brood stock were artificially spawned and the eggs were fertilized. A microinjection needle was loaded with the injection solution then connected to a microinjector. Injection volume was determined with a hemocytometer by injecting into a drop of mineral oil. Microinjection was performed by introducing the needle into the yolk and expelling the injection material. Embryos were then incubated in Holtfreter's solution until hatch. Indels in TICAM 1 and RBL genes were confirmed by DNA sequencing. Dramatic changes in the predicted protein sequence included frameshift and truncated protein due to a premature stop codon.

The effects of microinjection of different dosages of guide RNA (gRNA)/Cas9 protein on the mutation rate, embryo mortality, hatchability and early fry survival were investigated in channel catfish. Three dosages of gRNA/Cas9 protein (low, 2.5 ng gRNA/7.5 ng Cas9 protein, medium, 5 ng gRNA/15 ng Cas9 protein and high, 7.5 ng gRNA/22.5 ng Cas9 protein) targeting TICAM 1 and RBL genes, were compared. Microinjection increased the embryo mortality compared to non-injected controls. Injection of gRNA/Cas9 protein increased the embryo mortality when compared to the injected control embryos. Hatching percent was reduced with

injection of higher dosages of gRNA/Cas9 protein. Increasing the dosage of gRNA/Cas9 protein increased the mutation rate. Mutation rate was higher in dead embryos than 4-month old fingerlings, suggesting that off-target effects caused some mortality or knockout of the target genes affected viability. These experiments lay the foundations for designing and conducting gene editing experiments in channel catfish and can be used as a guide for other fish species.

A third study evaluated the efficacy of interspecific hybridization of channel catfish females and blue catfish *I. furcatus* males (CB hybrid catfish), and the transfer of cecropin B gene in enhancing the resistance to *Ichthyophthirius multifiliis* in catfish fingerlings and food size fish using a communal challenge. At the fingerling stage, cecropin transgenic channel catfish, cecropin transgenic CB hybrid catfish and non-transgenic CB hybrid catfish had similar resistance to ich and the three genotypes had significantly improved resistance to ich when compared to non-transgenic channel catfish. The experiment at the food size stage was conducted on non-transgenic channel catfish and non-transgenic CB hybrid catfish. CB hybrid catfish had a lower infection rate and higher survival rate than channel catfish. The results suggest that genetic enhancement of resistance to ich in catfish can be achieved by either generation of cecropin transgenic channel catfish or production of non-transgenic CB hybrid catfish, but no additional benefit was obtained by applying both genetic improvement programs simultaneously.

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List of Abbreviations

AGD   Amoebic gill disease

BCWD   Bacterial cold water disease

Cas9   CRISPR-associated protein 9

CB hybrid Channel catfish, *Ictalurus punctatus*, female x blue catfish male, *I. furcatus*, hybrid

CCV   Channel catfish virus

CRISPR   Clustered Regulatory Interspaced Short Palindromic Repeats

DSB   Double strand break

ERM   Enteric redmouth disease

ESC   Enteric Septicemia of Catfish

GCHV   Grass carp hemorrhage virus

GFP   Green fluorescent protein

gRNA   Guide RNA

HEW   Hen egg white

HIV-1   Human immunodeficiency virus 1

IHNV   Infectious hemopoietic necrosis virus

IPN   Infectious pancreatic necrosis

ISA   Infectious salmon anemia

KHV-E   Koi herpesvirus isolate E

LEAP-1   Liver-expressed antimicrobial peptide 1

LFB   Bovine lactoferrin

LHRHa   Luteinizing hormone releasing hormone analog

Mx   Myxovirus resistance proteins

NHEJ   Non-homologous end joining

PDG   Proliferative gill disease

RBL   Rhamnose binding lectin

RTFS   Rainbow trout fry syndrome

TALEN   Transcription Activator-Like Effector Nucleases

TICAM 1 Toll/interleukin 1 receptor domain-containing adapter molecule

VHS   Viral hemorrhagic septicemia

VHSV   Viral hemorrhagic septicemia virus

Chapter 1: Introduction

Aquaculture continues to grow significantly as a food production sector providing consumers with healthy food, creating more jobs and investment opportunities and enhancing food security. In 2014, inland and marine aquaculture production was 73.8 million tonnes, an increase of more than 20% compared to 2009, and the first-sale value was estimated to be more than US$ 160 billion in 2014 (FAO, 2016). Finfish production reached 49.8 million tonnes accounting for 67.5 % of world aquaculture production (FAO, 2016). In the US, 150 thousand tonnes of catfish were produced in 2014, a 54% decrease in production since the peak of 2003 (Hanson and Sites, 2015). To restore the peak of catfish production and to enhance its profitability, integration of multiple factors will be necessary, including management of catfish production operations, stimulating the immune system, disease prevention and genetic improvement of disease resistance and other traits.

Disease resistance is a critical trait as 40% of all aquaculture production (Owens, 2012) and 40% of catfish production (Tucker, 2012) is lost to disease. The losses from disease outbreaks result in mortality, lowered production, poor feed conversion ratios and financial loss from the cost of treatment and disease control measures. Bacterial disease cases dominated the total number of submitted cases in previous years (MSU, 2012) in the catfish industry. The most common bacterial infections in catfish are enteric septicemia of catfish (ESC) (*Edwardsiella ictaluri*) and columnaris (*Flavobacterium columnare*) (Plumb and Hanson, 2011). Losses in the channel catfish (*Ictalurus punctatus*) industry from *Flavobacterium columnare* and *Edwardsiella ictaluri* epizootics were estimated to be $60 – 80 million (Sommerset et al., 2014). Here,

different approaches to control diseases in aquaculture including chemotherapy, vaccination and genetic improvement programs are reviewed and discussed.

Chemotherapy

Several antibiotics and chemical compounds have been approved for aquaculture use such as florfenicol, oxytetracycline dihydrate and formalin (FDA, 2016). However, there are potential risks associated with chemotherapy in aquaculture. Risk of the bacteria developing antibiotic resistance will make that antibiotic less effective for curing diseases caused by antibiotic resistant bacteria. In addition, antibiotic resistant genes may be transferred to other bacterial species (Courvalin, 1994, Bennett, 2008). Antibiotic resistance was reported in *Vibrio anguillarum* (Aoki et al., 1981), *Flavobacterium psychrophilum* (Ekman, 2003) and *Flavobacterium columnare* (Declercq et al., 2013). These chemicals may also result in a bioaccumulation hazard, environmental pollution and risks to fish farmers who apply them. Costs and difficulties of application in aquaculture enterprises cannot be ignored and, in spite of being effective in reducing fish mortality (Soto et al., 2013, Gaunt et al., 2015), this approach does not provide a long term solution to the disease problems.

Vaccination

Vaccines are used to stimulate the immune system of the fish to provide protection against pathogens. Commercial vaccines are now available for several aquaculture fish species (Sommerset et al., 2014). For channel catfish, two live attenuated vaccines for *Flavobacterium columnare* and *Edwardsiella ictaluri* are now commercially available. Research has been conducted to develop a more efficient vaccine for *Flavobacterium columnare* than the one

currently used (Mohammed, 2015). However, no commercial vaccines for other bacterial, viral and parasitic diseases are available. Viral vaccines are usually inactivated viruses, viral proteins or DNA vaccines with viral nucleic acids sequences (Sommerset et al., 2014). The two main methods of vaccine delivery to fish are immersion and injection. Immersion is available only for a small number of bacterial pathogens and difficult to use for large fish. Injection is more laborious, requires the fish to be of a certain size which makes it impossible to vaccinate fry or large numbers of fish with this technique. Additional shortcomings of vaccination include handling stress, costs of vaccines and immunity from vaccination may protect the fish for the short-term only.

Genetic approaches to improve disease resistance

Genetic improvement of disease resistance can be achieved through short-term breeding programs, long-term breeding programs or a combination of both. The improvement in resistance can be inherited by future generations, which will provide early protection for the fish at the fry stage when the immune system is not completely developed (Zapata et al., 1996). Genetic improvement of disease resistance can be achieved through selection, crossbreeding, interspecific hybridization, transgenesis and marker-assisted selection (Fuji et al., 2007, Dunham, 2011). Genome editing technology opens avenues for manipulating disease resistance genes for the benefit of aquaculture, but the research is still in its early stages. The benefits from genetic improvement programs can be maximized by integrating molecular genetic tools with traditional breeding programs (Dunham, 2011).

Selection

Selection is a long-term genetic improvement program that can be used to improve quantitative traits such as growth rate and disease resistance over a few to several generations. Utilization of the genetic variation of the resistance to diseases within and between populations needs to be considered to achieve the best possible improvement (Price, 1985). The response to selection is variable and depends upon the heritability of the trait of interest, phenotypic variation and selection intensity (Dunham, 2011). Different strategies for performing selection in fish exist. In mass or individual selection, individual fish are selected based on their own performance while in family selection, individuals are selected based on the mean of the performance of their families. The two approaches can be combined by selecting the best individuals from the best families to maximize and accelerate the rate of improvement (Dunham, 2011).

Selection for disease resistance has been implemented in several aquaculture species with variable outcomes. In salmonids, selection for enhancing the disease resistance has been successful. One of the first documented successful trials was for brook trout (*Salvelinus fontinalis*) selected from a population with endemic furunculosis where three generations of selection improved the survival from 2% in the original population to 69% in the selected population (Embody and Hayford, 1925). Selection for the resistance to furunculosis in brook trout and brown trout (*Salmo trutta*) improved the resistance (Ehlinger, 1964), however, one of the selected strains of brook trout was more susceptible to gill disease (Ehlinger, 1977). Recently, Henryon et al. (2005) detected additive genetic variation for resistance to enteric redmouth disease (ERM), rainbow trout fry syndrome (RTFS) and viral hemorrhagic septicemia

(VHS) demonstrating that selection would improve the resistance to these diseases in rainbow trout (*Oncorhynchus mykiss*). The resistance to the causative agents of furunculosis, infectious salmon anemia (ISA) and infectious pancreatic necrosis (IPN) in Atlantic salmon (*Salmo salar*) was estimated to be moderate to highly heritable indicating that the resistance to these diseases would be improved with selection (Ødegård et al., 2007, Wetten et al., 2007, Kjøglum et al., 2008). Strains of rainbow trout that are resistant to IPN (Okamoto et al., 1993) and bacterial cold water disease (BCWD) (Leeds et al., 2010) have been developed. In Krasnodar common carp (*Cyprinus carpio*), selection improved the resistance to dropsy (Kirpichnikov et al., 1993).

Genetic variation of disease resistance has been reported in catfish suggesting the possibility of improving the resistance to diseases through selection (Wolters and Johnson, 1994). However, in Kansas strain of channel catfish, the resistance to enteric septicemia of catfish (ESC) was not improved with one generation selection when the offspring of parents that survived an ESC outbreak were compared to offspring of Kansas select and Kansas random parents that had not been exposed to *Edwardsiella ictaluri* (Dunham et al., 1994). Mass selection for resistance to *Aeromonas hydrophila* in walking catfish (*Clarias macrocephalus*) did not improve the survival rate and time when the selected and control fish were disease challenged (Na-Nakorn et al., 1995).

Bosworth et al. (2003) compared the resistance to proliferative gill disease (PGD) in USDA 103-line channel catfish full sib families, channel catfish female x blue catfish male (*Ictalurus furcatus*) (CB) backcross hybrids and blue catfish mixed families and concluded that a genetic component for the resistance to PGD existed and the application of selection to improve the resistance to PGD may be possible. Research has been conducted on NWAC103 channel

catfish strain (Wolters et al., 2002) to incorporate multiple economically important traits such as growth, carcass yield and resistance to ESC using multitrait selection. In two generations of family selection, resistance to ESC was improved by 10.3% in the newly selected strain, USDA303 (Bilodeau-Bourgeois et al., 2007).

Marker-assisted selection and quantitative trait loci

Marker-assisted selection (MAS) is useful for indirect selection of traits that are difficult or expensive to measure, have low heritability, or require rearing of the fish until maturity such as carcass traits, fecundity and disease resistance. The use of different markers in selection for disease resistance would reduce the costs for challenge testing, keep valuable genotypes that may be lost by disease challenge and avoid the selection of potential disease carriers (Vike et al., 2009). The application of marker-assisted selection in aquaculture would not only accelerate the selection for disease resistance traits but also reduce the negative impacts of selection on other traits (Ødegård et al., 2011). Several disease resistance markers have been identified and could be used for selection such as quantitative trait loci (QTL) (Moen et al., 2009, Geng et al., 2015, Liu et al., 2016a), candidate genes for disease resistance (Fuji et al., 2006, Johnson et al., 2008) and gene expression profiling (Baoprasertkul et al., 2006, Thongda et al., 2014).

QTLs for disease resistance have been identified in several aquaculture species (Ozaki et al., 2001, Moen et al., 2007, Rodríguez-Ramilo et al., 2011). In rainbow trout, major QTLs for resistance/susceptibility to IPNV and whirling disease have been identified (Ozaki et al., 2001, Baerwald et al., 2011). In Atlantic salmon, QTLs for IPNV and ISA have been detected (Moen et al., 2007, Houston et al., 2008). In addition, QTLs have been identified for resistance to *Aeromonas salmonicida* and VHS in turbot (*Scophthalmus maximus*) (Rodríguez-Ramilo et al.,

2011, Rodríguez-Ramilo et al., 2014), resistance to fish pasteurellosis in gilthead sea bream (*Sparus aurata*) (Massault et al., 2011), resistance to *Vibrio anguillarum* in Japanese flounder (*Paralichthys olivaceus*) (Zhou et al., 2016), and ESC and columnaris disease resistance in channel catfish (Geng et al., 2015, Zhou et al., 2016).

The application of MAS in breeding programs has been successful for disease resistance in Japanese flounder. A microsatellite allele, Poli9-8TUF, was identified to control the resistance to lymphocystis disease (LD) in Japanese flounder (Fuji et al., 2006). Selective breeding for Poli9-8TUF reduced the incidence of LD in a selectively bred Japanese flounder population to zero when tested at two LD endemic farms compared to control population which had 4.5% and 6.3% LD incidences in the two farms (Fuji et al., 2007). MAS-lymphocystis disease resistant flounder are now commercially sold in Japan with 35% market penetration rate (Ozaki et al., 2012).

Intraspecific Crossbreeding

Crossbreeding is a short-term genetic improvement program in which unrelated individuals are crossed to achieve heterosis or hybrid vigor in a single generation. The improvement of disease resistance in crossbred F_1 fish has been successful in $50 – 70$ % of the crosses examined (Dunham, 2011). In 90% of Nile tilapia (*Oreochromis niloticus*) crosses, crossbred fry had heterotic survival of 90% when compared to the parents which had 60% survival (Dunham, 2011).

The crossing of 2 catfish strains at Auburn University (AU-M and AU-K) resulted in a fast growing crossbred catfish (AU-MxK) which exhibited heterosis for disease resistance

(Dunham and Smitherman, 1985, Padi, 2003). Using a diallel cross, Wolters and Johnson (1995) estimated the effects of crossing three channel catfish strains, Red River, Norris and Marion X Kansas (MK) on the resistance to *Edwardsiella ictaluri*. Following immersion challenge with *E. ictaluri*, the cross between Norris and MK had the highest survival rate ± SE (90.0 ± 1.5) when compared to Norris X Norris and MK X MK which had 52.5 ± 7.3 and 68.9 ± 2.6 mean survival rate ± SE, respectively. Crossbreeding of local strains of Gunther's walking catfish in Thailand improved the resistance to *Aeromonas hydrophila* in the crossbred offspring (Prarom, 1990). If the breeder is fortunate, crossbreeding will improve more than one trait. Crossbreeds of Marion female X Kansas male strains of channel catfish exhibit heterosis for multiple traits including growth, early sexual maturity and resistance to diseases (Dunham, 2011). Crossbreeding usually improves reproductive performance. In addition to improved growth and disease resistance, AU-M and AU-K crossbred channel catfish exhibited heterosis for early sexual maturity and higher spawning rates when three years old compared to their parents (Dunham and Smitherman, 1985).

Interspecific hybridization

Interspecific hybridization is the mating of different species to produce hybrids. The success rate of this approach is relatively high for disease resistance improvement (Dunham, 2011). In salmonids, brook trout X rainbow trout hybrids proved to be resistant to viral hemorrhagic septicemia virus (VHSV) and infectious hemopoietic necrosis virus (IHNV) (Dorson et al., 1991). Hybrids of brown trout female X Atlantic salmon male exhibited intermediate resistance to skin fluke, *Gyrodactylus salaris*, (Bakke et al., 1999) and significant heterosis for amoebic gill disease (AGD) resistance with the resistance improved by 69% over

the mid-parent average (Maynard et al., 2016). However, Atlantic salmon X Arctic char (*Salvelinus alpinus*) hybrids did not exhibit any improvement in the resistance to sea louse, *Lepeophtheirus salmonis*, when compared to the parents (Fleming et al., 2014).

Reciprocal hybrids of *Xiphophorus maculatus* and *Xiphophorus variatus* had significantly lower infection levels for *Ichthyophthirius multifiliis* when compared to the parental stock (Clayton and Price, 1994). Koi (*Cyprinus carpio*) X goldfish (*Carassius auratus*) hybrids had reduced mortality (35%) when challenged with koi herpesvirus isolate E (KHV-E) compared to koi X crucian carp hybrids (91% mortality) and koi without any history of disease (100% mortality) (Bergmann et al., 2010). In tilapia, resistance to *Aeromonas sobria* was improved in hybrids produced by crossing Nile tilapia (*Oreochromis niloticus*) female X blue tilapia (*Oreochromis aureus*) male (Cai et al., 2004).

Of almost 50 North American catfish interspecific hybrids, only the hybrid between channel catfish females and blue catfish males (CB hybrid) possesses superior economically important traits compared to both parent species (Smitherman and Dunham, 1985). CB hybrid catfish proved to be superior in growth, resistance to many pathogens, survival and production in high density ponds, harvestibility, dress out percentage and tolerance to low dissolved oxygen levels (Dunham and Masser, 2012).

CB hybrid catfish exhibited intermediate resistance to *Edwardsiella ictaluri* when compared to both parent species (Wolters et al., 1996) as blue catfish are almost totally resistant to this bacterial pathogen. Survival and antibody levels following *E. ictaluri* exposure were also intermediate between channel and blue catfish. The resistance to columnaris disease can be improved by the production of CB hybrid catfish (Dunham and Masser, 2012). When challenged

with *Flavobacterium columnare* BGFS-27 (genomovar II), CB hybrid catfish had 31% mortality and the onset of the disease lagged for 3 days while the two parental species, channel and blue catfish, had ~80% mortality (Arias et al., 2012). Dunham et al. (1990) also found that the hybrid had much greater resistance to columnaris than channel catfish in the pond environment during the fingerling stage.

Interspecific hybridization variably improved the resistance to channel catfish virus (CCV), *Ichthyophthirius multifiliis* or external parasites. The CB hybrid catfish had similar mortality rate as the channel catfish USDA 103-line when challenged with CCV (Silverstein et al., 2008). Xu et al. (2011) compared the resistance of channel catfish, blue catfish and CB hybrid catfish to *Ichthyophthirius multifiliis* where CB hybrid catfish were as susceptible to ich infections as channel catfish or blue catfish. However, the mean survival of the hybrid was about 10% higher, which would have been significantly different given greater replication. Griffin et al. (2014) found that the resistance to the digenetic trematode *Bolbophorus damnificus* in CB hybrid catfish was similar to channel catfish and blue catfish.

Transgenesis

Transgenic fish have been generated for several goals such as improvement of growth rate (Du et al., 1992), cold tolerance (Wang et al., 1995), generation of models to study human diseases (reviewed in Dooley and Zon, 2000), enhancement of disease resistance (Dunham et al., 2002), generation of novel ornamental fish (Gong et al., 2003), production of pharmaceutical proteins (Morita et al., 2004), monitoring environmental pollution (Amanuma et al., 2000, Cachot et al., 2007), modification of body composition (Yoshizaki et al., 2007, Cheng et al., 2014), transgenic sterilization (Su, 2012), and studying gene functions *in vivo* (Xu et al., 2003).

Regarding disease resistance, several transgenic fish expressing different genes have been produced.

Antimicrobial peptides

Cecropin

Cecropins are antimicrobial peptides that lyse cell membranes of bacteria causing their death (Moore et al., 1996). Cecropins were first discovered in the hemolymph of the giant silkmoth, *Hyalophora cecropia* (Stciner et al., 1981) before being identified in many other organisms (Hultmark et al., 1982, Lee et al., 1989, Kylsten et al., 1990, Tryselius et al., 1992). Cecropins are more effective against gram-negative than gram-positive bacteria (Moore et al., 1996). Resistance to bacterial diseases was improved two- to four-fold by generation of F_1 cecropin B transgenic channel catfish (Dunham et al., 2002). Introduction of cecropin gene into the medaka (*Oryzias latipes*) genome enhanced the resistance to *Pseudomonas fluorescens* and *Vibrio anguillarum* (Sarmasik et al., 2002).

Although, cecropin is an antibacterial protein, it can provide protection against other pathogens such as viruses, fungi and protozoa. Resistance to infection by *Aeromonas salmonicida* and IHNV have been improved in homozygous cecropin P1 and CF-17 transgenic rainbow trout (Chiou et al., 2014). Cecropins inhibited the replication of human immunodeficiency virus 1 (HIV-1) by suppressing viral gene expression (Wachinger et al., 1998).

Cecropins also exhibited a strong antifungal activity (Ekengren and Hultmark, 1999, Ji et al., 2014) against different fungal species including *Aspergillus, Fusarium* (De Lucca et al.,

1998, De Lucca et al., 2000) and *Candida* species (Park et al., 1997) *in vitro*. Additionally, antiprotozoal activity was observed against *Plasmodium* and *Leishmania* species where the normal development of oocysts was reduced or inhibited (Boman et al., 1989, Gwadz et al., 1989, Akuffo et al., 1998). Expression of cecropin A in cecropin transgenic mosquito *Anopheles gambiae* reduced *Plasmodium berghei* oocyst production by 60% when compared to non-transgenic mosquitos (Kim et al., 2004). The broad spectrum action of cecropins against a wide range of pathogens was explained by the ability of cecropins to form large time-variable and voltage-dependent ion channels in the lipid membranes that have similar structure in different organisms (Christensen et al., 1988, Milani et al., 2009).

Lactoferrin

Lactoferrin is an 80 kDa protein from the transferrin family that is responsible for binding, transport and metabolism of iron ions (Iyer and Lonnerdal, 1993). Lactoferrin is secreted in various body fluids mainly milk and other biological fluids with multiple functions (Lönnerdal and Iyer, 1995, García-Montoya et al., 2012). Lactoferrin has been transferred and expressed in several animal species (Chen et al., 2008, Meng et al., 2013, Cooper et al., 2015). Lactoferrin has broad spectrum antibacterial (Bellamy et al., 1992, Yamauchi et al., 1993), antiviral (Van der Strate et al., 2001), antiparasitic (Turchany et al., 1995, OMATA et al., 2001) and antifungal activity (Bellamy et al., 1994).

Transfer of human lactoferrin driven by carp β-actin promoter to grass carp (*Ctenopharyngodon idellus*) delayed the onset of clinical signs in grass carp fry when injected with grass carp hemorrhage virus (GCHV) (Zhong et al., 2002). Lactoferrin transgenic grass carp also exhibited enhanced resistance to *Aeromonas hydrophila* with improved phagocytic activity

and a high rate of bacterial clearance while serum lysozyme activity was not changed when compared to non-transgenic fish (Weifeng et al., 2004). Lin et al. (2010)   generated transgenic zebrafish containing bovine lactoferrin (LFB) gene fused with green fluorescent protein (GFP) in which gene expression was driven by zebrafish β-actin promoter. The functional domain of LFB was released exhibiting *in vitro* antibacterial activity against *Escherichia coli*, *Edwardsiella tarda* and *Aeromonas hydrophila*. Feeding the non-transgenic adult zebrafish with LFB transgenic embryos enhanced the resistance to *E. tarda* when compared to adult zebrafish fed non-transgenic embryos.

Hepcidin

Hepcidin is the liver-expressed antimicrobial peptide 1 (LEAP-1) that has been identified in human, animal and fish species (Krause et al., 2000, Pigeon et al., 2001, Shike et al., 2004). Hepcidin is involved in immunity against bacteria (Bao et al., 2005, Tao et al., 2014), viruses (Chia et al., 2010, Wang et al., 2010) and in iron metabolism (Hu et al., 2007). The effects of transfer of hepcidin gene to fish have been studied in transgenic zebrafish. Generation of transgenic zebrafish expressing *Oreochromis mossambicus* hepcidin 2-3 (TH2-3) gene increased the resistance to *Vibrio vulnificus* (204) strain, but not *Streptococcus agalactiae* (Hsieh et al., 2010) while the expression of *Oreochromis mossambicus* hepcidin 1-5 (TH1-5) and shrimp chelonianin in transgenic zebrafish enhanced the resistance against both pathogens *Vibrio vulnificus* and *Streptococcus agalactiae* (Pan et al., 2011). In both TH2-3 and TH1-5 transgenic zebrafish, the expression level of several of the immune-related genes was modulated as some of them were upregulated and some were downregulated.

Epinecidin-1

Epinecidin-1 is another fish antimicrobial peptide that has been identified in grouper (*Epinephelus coioides*) (Pan et al., 2007). Epinecidin-1 exhibits antibacterial, antifungal and antiprotozoal activities (Pan et al., 2009). Peng et al. (2010) developed a zebrafish transgenic line expressing epinecidin-1 using the Tol2 transposon system. When injected with *Vibrio vulnificus* and *Streptococcus agalactiae*, bacterial growth was reduced or inhibited in epinecidin-1 transgenic zebrafish tissues for up to 24 hours when compared to non-transgenic controls. Feeding groupers and zebrafish on diets containing recombinant epinecidin-1 from E. coli for 30 days resulted in modulation of immune-related gene expression and significant reduction of mortality in treated fish when challenged with *Vibrio vulnificus* compared to the controls (Pan et al., 2012). Epinecidin-1 was electrotransferred to skeletal muscles of grouper in two steps, injection of epinecidin-1 expressing plasmid into skeletal muscles then electroporation (Lee et al., 2013). When intramuscular injection of treatment and control groupers with *Vibrio vulnificus* 48 hours after electroporation was performed, treatment fish had significantly lower bacterial numbers and higher immune-related gene expression in their muscles when compared to controls.

Myxovirus resistance proteins

Myxovirus resistance (Mx) proteins are important for antiviral immunity (Haller and Kochs, 2011). They proved to be effective for enhancing the resistance to viruses in transgenic animals (Pavlovic et al., 1995) and fish (Su et al., 2009). Mx proteins inhibit viral replication (Caipang et al., 2003). Over expression of rare minnow (*Gobiocypris rarus*) Mx-like gene in Mx-transgenic rare minnow enhanced the resistance to grass carp reovirus (Su et al., 2009).

Lysozyme

Lysozyme is an antibacterial enzyme that attacks peptidoglycan in cell walls of bacteria (Nakamura et al., 1990). Thus, it is thought to be more effective against Gram positive bacteria than Gram negative bacteria. Yazawa et al. (2006) transferred the hen egg white (HEW) lysozyme gene and GFP gene driven by Japanese flounder keratin promoter to zebrafish. Gene expression was confirmed in the liver and skin. Increased lytic activity in the protein extracts from the liver of F_2 transgenic fish was found to be 1.75 times higher than wild type fish. The survival after *Edwardsiella tarda* and *Flavobacterium columnare* challenge was improved in F_2 transgenic zebrafish (60% and 65% survival respectively) compared to 0% survival in wild type fish. In Atlantic salmon, Fletcher et al. (2011) generated a stable transgenic salmon line that was expressing lysozyme under the control of ocean pout antifreeze protein gene promoter op5a. The lytic activity kidney tissue extracts from F_2 lysozyme transgenic fish showed 40% increase when compared to wildtype siblings. However, the evaluation of disease resistance in lysozyme transgenic Atlantic salmon to actual challenge with bacteria was not reported.

The approach for improving the disease resistance in fish by the transfer of lysozyme gene may not be as effective as other antimicrobial peptide genes. Most economically important diseases in aquaculture are caused by Gram negative bacteria. It has been reported that different bacterial species were able to evade the lysozyme-based peptidoglycan resulting in the emergence of lysozyme resistant bacteria (Schwartz et al., 2003, Bera et al., 2005, Shimada et al., 2010). It appears that the effectiveness of higher lysozyme activity in conveying disease resistance is greatly affected by the immune status of the fish (Røed et al., 2002). Experimental challenge of high- and low-lysozyme lines of rainbow trout with *Aeromonas salmonicida* and

Vibrio anguillarum yielded different results. Before vaccination, mortality due to *A. salmonicida* was higher in the high-lysozyme line while after vaccination, the high-lysozyme line had significantly higher mortality rate and higher levels of antibodies when compared to the low-lysozyme line. For *V. anguillarum* challenge, mortality was high in both lines before vaccination, while after vaccination, slight but not significant increase in survival and significant increase in antibody levels were seen in the high-lysozyme line when compared to the low-lysozyme line (Røed et al., 2002). In these experiments, bacteria were injected intraperitoneally, which eliminated the natural defense barriers, especially the mucosal barriers.

Pleiotropic effects of transgenes on disease resistance

The insertion of transgenes can result in pleiotropic effects (Dunham, 2011). Pleiotropic effects occur when a transgene affects more than one trait in a positive or a negative manner. Pleiotropic effects are discussed with emphasis on disease resistance. Transgenic common carp containing rainbow trout growth hormone had higher survival than non-transgenic controls when exposed to stressors and pathogens such as hypoxia, anchor worms, *Aeromonas* and dropsy (Chatakondi, 1995). Transfer and expression of "all-fish" growth hormone in common carp significantly enhanced non-specific immune functions in transgenic common carp when compared to non-transgenic carp (Wang et al., 2006). These immune functions included lysozyme activity, serum bactericidal activity, leukocrit and phagocytic percent of macrophages in head kidney which were significantly higher in the transgenic carp. However, phagocytic indices and relative spleen weight were not different in both genotypes (Wang et al., 2006). When challenged with *Ichthyophthirius multifiliis*, all-fish growth hormone transgenic common carp had higher survival in comparison to non-transgenic common carp (Ling et al., 2009).

Expression of delta-5 (Δ-5) desaturase and delta-6 (Δ-6) desaturase in the muscles of transgenic zebrafish not only increased the polyunsaturated fatty acid content but also enhanced the resistance to *Vibrio alginolyticus* infections as well as anti-inflammatory reactions (Wang et al., 2014). At 4 and 12 h after injection of *V. alginolyticus* into the muscles, transgenic zebrafish showed enhanced antibacterial activity and modulation of the expression of multiple cytokines (IL-1β, IL-22, and TNF-α) resulting in decreased inflammatory response.

On the other hand, growth hormone transgenic coho salmon (*Oncorhynchus kisutch*) were as resistant as non-transgenic fish when infected with *V. anguillarum* at the fry stage. However, transgenic fish were more susceptible to *V. anguillarum* infection than non-transgenic fish at the smolt stage (Jhingan et al., 2003). In these transgenic salmon fish, there was much family variation. Disease resistance was sometimes improved, reduced or unchanged when compared to non-transgenic controls.

Gene editing technology

Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) are genome editing tools that has been used to induce double strand breaks (DSB) in genes of interest. Depending upon how the cell addresses the DSB, gene knockout, knock-in (insertion) or correction may be achieved. If the DSB is repaired by non-homologous end joining (NHEJ), mutations (mostly indels) will occur. Genes could be inserted or corrected by providing a DNA template for DSB repair by homologous recombination (Urnov et al., 2005). ZFNs and TALENs utilize the phenomenon that DNA domains can bind to specific DNA sequence. When linked with a DNA cutting domain,

endonuclease, they can induce DSB. Both can be programmed to target a specific gene. The DNA binding domain will differ depending on the gene being targeted while the nuclease domain remains the same. The domains and efficiency are different from ZFNs to TALENs.

ZFNs:

DNA cleavage domains are from the *FokI* restriction endonuclease combined with zinc finger proteins (ZFP) (Porteus and Carroll, 2005). To induce DSB, ZFNs must be engineered in pairs, one for each DNA strand. Efficiency could be explained not only inducing the DSB efficiently, but also in terms of specificity and cytotoxicity. Off-target effects result when the ZFP binds to the non-intended DNA sequence and this could result in lethality (cytotoxicity) if essential genes were targeted in error (Pattanayak et al., 2011) or when the DSBs become too great for the cell of the organism to tolerate. Improved design has been made through the use of heterodimer variants of *FokI* which reduced DNA damage in mammalian cells (Miller et al., 2007). Zinc fingers have gene targeting frequencies of up to 20% (Porteus and Carroll, 2005). In channel catfish, the overall mutation rate for three ZFN sets was 19.7% with one ZFN set inducing average mutation rate of 38.5 % in two of the treatments (Qin et al., 2016). Because of greater costs, complexity of design, off-target effects, cytotoxicity and lower mutation rate, research on ZFNs is decreasing especially once TALENs became available.

TALENs:

TALENs also have two domains, the DNA binding domain (TALE) and nuclease domain (*FokI*). They can be engineered to target a specific DNA sequence with the Repeat Variable Di-residue (Boch, 2011) (Fig 2). TALENs share the same principle as ZFNs but they are less costly

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and more efficient in terms of specificity, off target effects and cytotoxicity. Off target effects were low compared to ZFNs when whole genome sequencing was performed (Veres et al., 2014). The mutation rate for TALENs gene edited zebrafish embryos ranged from 11% to 33% with small indels and in rare cases a deletion of more than 300 bp (Sander et al., 2011). In channel catfish, mutation rates ranged from 45% to 63% (Qin, 2015). Cytotoxicity was low due to high specificity when compared to ZFNs (Mussolino et al., 2014).

CRISPR/Cas9 Technology:

Clustered regularly interspaced short palindromic repeats (CRISPRs) were described in 2002 in bacteria and archea (Jansen et al., 2002) before being extensively studied and used for gene editing experiments. CRISPRs are 21-37 bp identical repeats that are separated from each other by a non-repetitive DNA sequence called Spacer DNA. The spacers are derived from viral genomic sequences (Barrangou et al., 2007) or plasmids (Garneau et al., 2010). CRISPR associated (Cas) genes are not present in all species of bacteria (Jansen et al., 2002).

CRISPRs play an important role in the bacterial immunity against phages and plasmids. Barrangou et al. (2007) found that different strains of *Streptococcus thermophiles* bacteria with different number and type of spacers have different levels of resistance to viruses. Addition or removal of a spacer modified the resistance against phages. Segments of viral genomic sequences were inserted into the bacterial CRISPR loci as a spacer sequence. This process may explain the "acquired immunity" of bacterial cells against phages to which they have been previously exposed (Al-Attar et al., 2011, Terns and Terns, 2011). Once the viral genomic sequence is processed and integrated into the CRISPR loci, bacteria become resistant to the phage from which the spacer DNA originated, provided that the phage does not evolve to escape

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bacterial defense. When viral DNA was inserted into the cell, it was attacked by the defense mechanism of bacterial cells.

The principle has been used to engineer CRISPR-Cas systems to target specific DNA sequence more efficiently than TALENs and ZFNs (Mali et al., 2013). The system consists of a guide RNA which determines the targeted sequence in the genome and a DNA endonuclease enzyme, Cas9. When introduced into the cell, Cas9 will induce DSB, and depending on the goal, genes can be mutated, corrected or inserted. Compared to ZFNs and TALENs, CRISPR/Cas9 system is preferred for its simplicity, efficiency, accuracy, cheaper cost, lower toxicity and higher germline transfer of mutations (Varshney et al., 2015).

Applications of CRISPR/Cas9 technology for disease resistance research in aquaculture

Functional genomics

The genome of channel catfish has been sequenced (Liu et al., 2016b), however, there are still many genes in which gene function is not clearly understood. There is an increasing importance to study gene function in channel catfish to identify candidate genes that are important for the development of a particular economically important trait. Understanding gene function can assist in selecting the best fish for commercial production as well as recreational fishing activities.

In progression of disease, expression of immune-related genes may become upregulated or downregulated and the expression level for the same gene may differ from one species to another (Baoprasertkul et al., 2006, Peatman et al., 2007). This genetic variation could be more thoroughly investigated to identify the putative roles of these genes and their correlation with

phenotypic variation for disease resistance. In channel catfish, which are more susceptible to *Edwardsiella ictaluri* infection, expression of toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene was dramatically upregulated following bacterial challenge with *E. ictaluri* while it was downregulated in blue catfish, a resistant species to *E. ictaluri* infection (Wolters and Johnson, 1994, Hawke et al., 1998, Baoprasertkul et al., 2006) . Therefore, knocking out TICAM 1 gene would increase the understanding of its function during the pathogenesis of ESC. Another gene whose expression is correlated to disease progression is the rhamnose binding lectin (RBL) gene. Following experimental infection with *F. columnare* in a columnaris susceptible strain of channel catfish, there was an acute robust upregulation of RBL gene expression when compared to a columnaris resistant strain of channel catfish (Beck et al., 2012). Blocking channel catfish RBL *in vivo* using L-rhamnose 15 minutes before a columnaris challenge reduced the mortality in treatment groups compared to controls (Beck et al., 2012). Many other genes have been reported to be differentially regulated following *E. ictaluri* (Peatman et al., 2007, Peatman et al., 2008), *F. columnare* (Sun et al., 2012) and *A. hydrophila* (Li et al., 2013) infections. Once potential genes are identified, they can be selected for or against to genetically improve aquatic organisms for the benefit of aquaculture.

Targeted gene insertion

Transgenic fish resistant to infectious diseases have been generated through the introduction of transgenes using different techniques such as embryo microinjection, embryo electroporation and sperm-mediated gene transfer. Once transgenes are introduced, and regardless of the technique, there is no control on how many transgene copies and where in the genome transgene integration will occur. Integration of transgenes has been reported to be

random and to occur at multiple integration sites with multiple copies at the same locus with different concatameric arrangements (Tewari et al., 1992, Dunham, 2011). Although the time and tissue of transgene expression are controlled by the promoter, integration site, whether it is heterochromaic or euchromatic, has been shown to affect the transgene expression (Robertson et al., 1995, Dobie et al., 1996). Yan et al. (2006) reported that histone modifications can allow or inhibit transgene expression. A chromatin region is permissive to transgene expression when it is enriched in histone H3K4 methylation and H3 acetylation while non-permissive chromatin regions are poor or depleted of these modifications.

High copy numbers of transgenes were integrated e.g. about 100 copies of a transgene were detected per germ cell of a transgenic zebrafish (Stuart et al., 1988). The effects of transgene copy number on the level of expression were variable. Integration of multiple concatameric homologous copies of a transgene, which is common in transgenic fish, has been reported to repress gene expression in plants (Linn et al., 1990, Assaad et al., 1993), drosophila (Dorer and Henikoff, 1994, Sabl and Henikoff, 1996) and mammals (Garrick et al., 1998). Other researchers reported increased transgene expression with increased copy numbers (Grosveld et al., 1987, Hobbs et al., 1993, Tang et al., 2003). With random integration of uncontrolled copies of transgenes, a large number of fish will need to be tested in a time consuming, expensive manner to identify founder fish with the desired transgene integration site and the intended expression level.

Genome editing tools provide the ability to "knockin" or insert transgenes at specific loci. A donor DNA template is co-injected with CRISPR/Cas9 into one cell embryos. The donor template contains the transgene flanked by a DNA sequence that is homologous to the loci in

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which gene insertion is intended. In this case, homologous recombination machinery will use the provided DNA template to correct the DSB which will result in the insertion of one copy of the transgene at the target locus (Auer et al., 2014). This strategy has some limitations, including and especially the efficiency. The rate of transgene insertion depends on the repair mechanism of the DSB. In mammalian cells, DSB are mostly repaired by the NHEJ pathway (Mao et al., 2008). However, successful trials of switching the DSB repair mechanism from NHEJ to homologous recombination by suppressing NHEJ key molecules such as KU70, KU80 and DNA ligase IV have been reported (Chu et al., 2015).

Objectives:

1. Develop and validate a microinjection protocol for gene editing in channel catfish using CRISPR/Cas9 technology. Successful knockout of two immune-related genes, TICAM1 gene and RBL gene was achieved. The protocol can be modified to microinject transgene constructs, insert or correct genes or regulate gene expression using CRISPR/Cas9 technology.

2. Assess the effects of microinjection of different dosages of gRNAs/Cas9 protein on the mutation rate, embryo mortality, hatch and early fry survival. This study was conducted by targeting TICAM and RBL genes which will help identify the optimum dosage to achieve the best mutation rate with less adverse effects on the embryos.

3. Evaluate cecropin transgenesis and interspecific hybridization of channel catfish female X blue catfish male as genetic improvement programs to enhance the resistance to *Ichthyophthirius multifiliis* infections at the fingerling and food size stages.

References

Akuffo, H., Hultmark, D., Engstöm, A., Frohlich, D. & Kimbrell, D. (1998). Drosophila antibacterial protein, cecropin A, differentially affects non-bacterial organisms such as Leishmania in a manner different from other amphipathic peptides. *International journal of molecular medicine,* 1**:** 77-159.

Al-Attar, S., Westra, E.R., Van Der Oost, J. & Brouns, S.J. (2011). Clustered regularly interspaced short palindromic repeats (CRISPRs): the hallmark of an ingenious antiviral defense mechanism in prokaryotes. *Biological chemistry,* 392**:** 277-289.

Amanuma, K., Takeda, H., Amanuma, H. & Aoki, Y. (2000). Transgenic zebrafish for detecting mutations caused by compounds in aquatic environments. *Nature biotechnology,* 18**:** 62-65.

Aoki, T., Kitao, T. & Kawano, K. (1981). Changes in drug resistance of *Vibrio anguillarum* in cultured ayu, *Plecoglossus altivelis* Temminck and Schlegel, in Japan. *Journal of Fish Diseases,* 4**:** 223-230.

Arias, C.R., Cai, W., Peatman, E. & Bullard, S.A. (2012). Catfish hybrid *Ictalurus punctatus*× *I. furcatus exhibits* higher resistance to columnaris disease than the parental species. *Diseases of Aquatic Organisms,* 100**:** 77-81.

Assaad, F.F., Tucker, K.L. & Signer, E.R. (1993). Epigenetic repeat-induced gene silencing (RIGS) inArabidopsis. *Plant molecular biology,* 22**:** 1067-1085.

Auer, T.O., Duroure, K., De Cian, A., Concordet, J.-P. & Del Bene, F. (2014). Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. *Genome research,* 24**:** 142-153.

Baerwald, M., Petersen, J., Hedrick, R., Schisler, G. & May, B. (2011). A major effect quantitative trait locus for whirling disease resistance identified in rainbow trout (*Oncorhynchus mykiss*). *Heredity,* 106**:** 920-926.

Bakke, T., Soleng, A. & Harris, P. (1999). The susceptibility of Atlantic salmon (*Salmo salar* L.)× brown trout (*Salmo trutta* L.) hybrids to *Gyrodactylus salaris* Malmberg and *Gyrodactylus derjavini* Mikailov. *Parasitology,* 119**:** 467-481.

Bao, B., Peatman, E., Li, P., He, C. & Liu, Z. (2005). Catfish hepcidin gene is expressed in a wide range of tissues and exhibits tissue-specific upregulation after bacterial infection. *Developmental & Comparative Immunology,* 29**:** 939-950.

Baoprasertkul, P., Peatman, E., Somridhivej, B. & Liu, Z. (2006). Toll-like receptor 3 and TICAM genes in catfish: species-specific expression profiles following infection with *Edwardsiella ictaluri*. *Immunogenetics,* 58**:** 817-830.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A. & Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science,* 315**:** 1709-1712.

Beck, B.H., Farmer, B.D., Straus, D.L., Li, C. & Peatman, E. (2012). Putative roles for a rhamnose binding lectin in *Flavobacterium columnare* pathogenesis in channel catfish *Ictalurus punctatus. Fish & shellfish immunology,* 33**:** 1008-1015.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K. & Tomita, M. (1992). Identification of the bactericidal domain of lactoferrin. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology,* 1121**:** 130-136.

Bellamy, W., Yamauchi, K., Wakabayashi, H., Takase, M., Takakura, N., Shimamura, S. & Tomita, M. (1994). Antifungal properties of lactoferricin B, a peptide derived from the N-terminal region of bovine lactoferrin. *Letters in applied microbiology,* 18**:** 230-233.

Bennett, P. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British journal of pharmacology,* 153**:** S347-S357.

Bera, A., Herbert, S., Jakob, A., Vollmer, W. & Götz, F. (2005). Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus. Molecular microbiology,* 55**:** 778-787.

Bergmann, S.M., Sadowski, J., Kiełpiński, M., Bartłomiejczyk, M., Fichtner, D., Riebe, R., Lenk, M. & Kempter, J. (2010). Susceptibility of koi× crucian carp and koi× goldfish hybrids to koi herpesvirus (KHV) and the development of KHV disease (KHVD). *Journal of fish diseases,* 33**:** 267-272.

Bilodeau-Bourgeois, A.L., Bosworth, B.G. & Wolters, W.R. (2007). Reductions in susceptibility of channel catfish, *Ictalurus punctatus*, to enteric septicemia of catfish through two generations of selection. *Journal of the World Aquaculture Society,* 38**:** 450-453.

Boch, J. (2011). TALEs of genome targeting. *Nature biotechnology,* 29**:** 135-136.

Boman, H., Wade, D., Boman, I., Wåhlin, B. & Merrifield, R. (1989). Antibacterial and antimalarial properties of peptides that are cecropin-melittin hybrids. *FEBS letters,* 259**:** 103-106.

Bosworth, B.G., Wise, D.J., Terhune, J.S. & Wolters, W.R. (2003). Family and genetic group effects for resistance to proliferative gill disease in channel catfish, blue catfish and channel catfish× blue catfish backcross hybrids. *Aquaculture Research,* 34**:** 569-573.

Cachot, J., Law, M., Pottier, D., Peluhet, L., Norris, M., Budzinski, H. & Winn, R. (2007). Characterization of toxic effects of sediment-associated organic pollutants using the λ transgenic medaka. *Environmental science & technology,* 41**:** 7830-7836.

Cai, W.-Q., Li, S.-F. & Ma, J.-Y. (2004). Diseases resistance of Nile tilapia (*Oreochromis niloticus*), blue tilapia (*Oreochromis aureus*) and their hybrid (female Nile tilapia× male blue tilapia) to *Aeromonas sobria*. *Aquaculture,* 229**:** 79-87.

Caipang, C.M.A., Hirono, I. & Aoki, T. (2003). In vitro inhibition of fish rhabdoviruses by Japanese flounder, *Paralichthys olivaceus* Mx. *Virology,* 317**:** 373-382.

Chatakondi, N. (1995). Evaluation of transgenic common carp, *Cyprinus carpio*, containing rainbow trout growth hormone in ponds. PhD dissertation, Auburn University, Auburn, Alabama, USA.

Chen, H.-L., Wang, L.-C., Chang, C.-H., Yen, C.-C., Cheng, W.T., Wu, S.-C., Hung, C.-M., Kuo, M.-F. & Chen, C.-M. (2008). Recombinant porcine lactoferrin expressed in the milk of transgenic mice protects neonatal mice from a lethal challenge with enterovirus type 71. *Vaccine,* 26**:** 891-898.

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

Chia, T.-J., Wu, Y.-C., Chen, J.-Y. & Chi, S.-C. (2010). Antimicrobial peptides (AMP) with antiviral activity against fish nodavirus. *Fish & shellfish immunology,* 28**:** 434-439.

Chiou, P.P., Chen, M.J., Lin, C.-M., Khoo, J., Larson, J., Holt, R., Leong, J.-A., Thorgarrd, G. & Chen, T.T. (2014). Production of homozygous transgenic rainbow trout with enhanced disease resistance. *Marine biotechnology,* 16**:** 299-308.

Christensen, B., Fink, J., Merrifield, R. & Mauzerall, D. (1988). Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. *Proceedings of the National Academy of Sciences,* 85**:** 5072-5076.

Chu, V.T., Weber, T., Wefers, B., Wurst, W., Sander, S., Rajewsky, K. & Kühn, R. (2015). Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nature biotechnology,* 33**:** 543-548.

Clayton, G. & Price, D. (1994). Heterosis in resistance to *Ichthyophthirius multifiliis* infections in poeciliid fish. *Journal of fish biology,* 44**:** 59-66.

Cooper, C.A., Maga, E.A. & Murray, J.D. (2015). Production of human lactoferrin and lysozyme in the milk of transgenic dairy animals: past, present, and future. *Transgenic research,* 24**:** 605-614.

Courvalin, P. (1994). Transfer of antibiotic resistance genes between gram-positive and gram-negative bacteria. *Antimicrobial agents and chemotherapy,* 38**:** 1447.

De Lucca, A., Bland, J., Jacks, T., Grimm, C. & Walsh, T. (1998). Fungicidal and binding properties of the natural peptides cecropin B and dermaseptin. *Medical mycology,* 36**:** 291-298.

De Lucca, A., Bland, J., Vigo, C., Jacks, T., Peter, J. & Walsh, T. (2000). D-cecropin B: proteolytic resistance, lethality for pathogenic fungi and binding properties. *Medical mycology,* 38**:** 301-308.

Declercq, A., Boyen, F., Den Broeck, W., Bossier, P., Karsi, A., Haesebrouck, F. & Decostere, A. (2013). Antimicrobial susceptibility pattern of *Flavobacterium columnare* isolates collected worldwide from 17 fish species. *Journal of fish diseases,* 36**:** 45-55.

Dobie, K.W., Lee, M., Fantes, J.A., Graham, E., Clark, A.J., Springbett, A., Lathe, R. & Mcclenaghan, M. (1996). Variegated transgene expression in mouse mammary gland is determined by the transgene integration locus. *Proceedings of the National Academy of Sciences,* 93**:** 6659-6664.

Dooley, K. & Zon, L.I. (2000). Zebrafish: a model system for the study of human disease. *Current opinion in genetics & development,* 10**:** 252-256.

Dorer, D.R. & Henikoff, S. (1994). Expansions of transgene repeats cause heterochromatin formation and gene silencing in Drosophila. *Cell,* 77**:** 993-1002.

Dorson, M., Chevassus, B. & Torhy, C. (1991). Comparative susceptibility of three species of char and of rainbow trout X char triploid hybrids to several pathogenic salmonid viruses. *Diseases of aquatic Organisms,* 11**:** 217-224.

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

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

Dunham, R.A., Brady, Y. & Vinitnantharat, S. (1994). Response to challenge with *Edwardsiella ictaluri* by channel catfish, *Ictalurus punctatus,* selected for resistance to *E. ictaluri*. *Journal of Applied Aquaculture,* 3**:** 211-222.

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

Dunham, R.A. & Masser, M.P. (2012). *Production of hybrid catfish*: Southern Regional Aquaculture Center.

Dunham, R.A. & Smitherman, R.O. (1985). Improved growth rate, reproductive performance, and disease resistance of crossbred and selected catfish from AU-M and AU-K lines.

Dunham, R.A., Warr, G.W., Nichols, A., Duncan, P.L., Argue, B., Middleton, D. & Kucuktas, H. (2002). Enhanced bacterial disease resistance of transgenic channel catfish *Ictalurus punctatus* possessing cecropin genes. *Marine biotechnology,* 4**:** 338-44. DOI 10.1007/s10126-002-0024-y

Ehlinger, N.F. (1964). Selective breeding of trout for resistance to furunculosis. *NY Fish Game J,* 11**:** 78-90.

Ehlinger, N.F. (1977). Selective breeding of trout for resistance to furunculosis. *NY Fish Game J,* 24**:** 25-36.

Ekengren, S. & Hultmark, D. (1999). Drosophila cecropin as an antifungal agent. *Insect biochemistry and molecular biology,* 29**:** 965-972.

Ekman, E. (2003). *Natural and experimental infections with Flavobacterium psychrophilum in salmonid fish.*

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

FAO (2016). The state of world fisheries and aquaculture. Contributing to food security and nutrition for all. Rome. 200 pp.

FDA (2016). Approved aquaculture drugs. Retrieved from http://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/Aquaculture/ucm132954.htm

Fleming, M., Hansen, T., Skulstad, O., Glover, K., Morton, C., Vøllestad, L. & Fjelldal, P. (2014). Hybrid salmonids: ploidy effect on skeletal meristic characteristics and sea lice infection susceptibility. *Journal of Applied Ichthyology,* 30**:** 746-752.

Fletcher, G.L., Hobbs, R.S., Evans, R.P., Shears, M.A., Hahn, A.L. & Hew, C.L. (2011). Lysozyme transgenic Atlantic salmon (*Salmo salar* L.). *Aquaculture Research,* 42**:** 427-440.

Fuji, K., Hasegawa, O., Honda, K., Kumasaka, K., Sakamoto, T. & Okamoto, N. (2007). Marker-assisted breeding of a lymphocystis disease-resistant Japanese flounder (Paralichthys olivaceus). *Aquaculture,* 272**:** 291-295.

Fuji, K., Kobayashi, K., Hasegawa, O., Coimbra, M.R.M., Sakamoto, T. & Okamoto, N. (2006). Identification of a single major genetic locus controlling the resistance to lymphocystis disease in Japanese flounder (*Paralichthys olivaceus*). *Aquaculture,* 254**:** 203-210.

García-Montoya, I.A., Cendón, T.S., Arévalo-Gallegos, S. & Rascón-Cruz, Q. (2012). Lactoferrin a multiple bioactive protein: an overview. *Biochimica et Biophysica Acta (BBA)-General Subjects,* 1820**:** 226-236.

Garneau, J.E., Dupuis, M.-È., Villion, M., Romero, D.A., Barrangou, R., Boyaval, P., Fremaux, C., Horvath, P., Magadán, A.H. & Moineau, S. (2010). The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature,* 468**:** 67-71.

Garrick, D., Fiering, S., Martin, D.I. & Whitelaw, E. (1998). Repeat-induced gene silencing in mammals. *Nature genetics,* 18**:** 56-59.

Gaunt, P.S., Chatakondi, N., Gao, D. & Endris, R. (2015). Efficacy of florfenicol for control of mortality associated with *Edwardsiella ictaluri* in three species of catfish. *Journal of aquatic animal health,* 27**:** 45-49.

Geng, X., Sha, J., Liu, S., Bao, L., Zhang, J., Wang, R., Yao, J., Li, C., Feng, J. & Sun, F. (2015). A genome-wide association study in catfish reveals the presence of functional hubs of related genes within QTLs for columnaris disease resistance. *BMC genomics,* 16**:** 1.

Gong, Z., Wan, H., Tay, T.L., Wang, H., Chen, M. & Yan, T. (2003). Development of transgenic fish for ornamental and bioreactor by strong expression of fluorescent proteins in the skeletal muscle. *Biochemical and biophysical research communications,* 308**:** 58-63.

Griffin, M.J., Reichley, S.R., Khoo, L.H., Ware, C., Greenway, T.E., Mischke, C.C. & Wise, D.J. (2014). Comparative susceptibility of Channel Catfish, Blue Catfish, and their hybrid cross to experimental challenge with *Bolbophorus damnificus* (Digenea: Bolbophoridae) cercariae. *Journal of aquatic animal health,* 26**:** 96-99.

Grosveld, F., Van Assendelft, G.B., Greaves, D.R. & Kollias, G. (1987). Position-independent, high-level expression of the human β-globin gene in transgenic mice. *Cell,* 51**:** 975-985.

Gwadz, R., Kaslow, D., Lee, J.-Y., Maloy, W., Zasloff, M. & Miller, L. (1989). Effects of magainins and cecropins on the sporogonic development of malaria parasites in mosquitoes. *Infection and Immunity,* 57**:** 2628-2633.

Haller, O. & Kochs, G. (2011). Human MxA protein: an interferon-induced dynamin-like GTPase with broad antiviral activity. *Journal of Interferon & Cytokine Research,* 31**:** 79-87.

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

Hawke, J.P., Durborow, R., Thune, R. & Camus, A. (1998). *ESC: enteric septicemia of catfish*: Southern Regional Aquaculture Center Stoneville, Mississippi.

Henryon, M., Berg, P., Olesen, N.J., Kjær, T.E., Slierendrecht, W.J., Jokumsen, A. & Lund, I. (2005). Selective breeding provides an approach to increase resistance of rainbow trout (*Onchorhynchus mykiss*) to the diseases, enteric redmouth disease, rainbow trout fry syndrome, and viral haemorrhagic septicaemia. *Aquaculture,* 250**:** 621-636.

Hobbs, S.L., Warkentin, T.D. & Delong, C.M. (1993). Transgene copy number can be positively or negatively associated with transgene expression. *Plant molecular biology,* 21**:** 17-26.

Houston, R.D., Haley, C.S., Hamilton, A., Guy, D.R., Tinch, A.E., Taggart, J.B., Mcandrew, B.J. & Bishop, S.C. (2008). Major quantitative trait loci affect resistance to infectious pancreatic necrosis in Atlantic salmon (*Salmo salar*). *Genetics,* 178**:** 1109-1115.

Hsieh, J.-C., Pan, C.-Y. & Chen, J.-Y. (2010). Tilapia hepcidin (TH) 2-3 as a transgene in transgenic fish enhances resistance to *Vibrio vulnificus* infection and causes variations in immune-related genes after infection by different bacterial species. *Fish & shellfish immunology,* 29**:** 430-439.

Hu, X., Camus, A.C., Aono, S., Morrison, E.E., Dennis, J., Nusbaum, K.E., Judd, R.L. & Shi, J. (2007). Channel catfish hepcidin expression in infection and anemia. *Comparative immunology, microbiology and infectious diseases,* 30**:** 55-69.

Hultmark, D., Engstrom, Å., Bennich, H., Kapur, R. & Boman, H.G. (1982). Insect immunity: isolation and structure of cecropin D and four minor antibacterial components from Cecropia pupae. *European journal of biochemistry,* 127**:** 207-217.

Iyer, S. & Lonnerdal, B. (1993). Lactoferrin, lactoferrin receptors and iron metabolism. *European journal of clinical nutrition*.

Jansen, R., Embden, J., Gaastra, W. & Schouls, L. (2002). Identification of genes that are associated with DNA repeats in prokaryotes. *Molecular microbiology,* 43**:** 1565-1575.

Jhingan, E., Devlin, R. & Iwama, G. (2003). Disease resistance, stress response and effects of triploidy in growth hormone transgenic coho salmon. *Journal of Fish Biology,* 63**:** 806-823.

Ji, S., Li, W., Zhang, L., Zhang, Y. & Cao, B. (2014). Cecropin A–melittin mutant with improved proteolytic stability and enhanced antimicrobial activity against bacteria and fungi associated with gastroenteritis in vitro. *Biochemical and biophysical research communications,* 451**:** 650-655.

Johnson, N.A., Vallejo, R.L., Silverstein, J.T., Welch, T.J., Wiens, G.D., Hallerman, E.M. & Palti, Y. (2008). Suggestive association of major histocompatibility IB genetic markers with resistance to bacterial cold water disease in rainbow trout (*Oncorhynchus mykiss*). *Marine biotechnology,* 10**:** 429-437.

Kim, W., Koo, H., Richman, A.M., Seeley, D., Vizioli, J., Klocko, A.D. & O'brochta, D.A. (2004). Ectopic expression of a cecropin transgene in the human malaria vector mosquito *Anopheles gambiae* (Diptera: Culicidae): effects on susceptibility to Plasmodium. *Journal of medical entomology,* 41**:** 447-455.

Kirpichnikov, V., Ilyasov, J.I., Shart, L., Vikhman, A., Ganchenko, M., Ostashevsky, A., Simonov, V., Tikhonov, G. & Tjurin, V. (1993). Selection of Krasnodar common carp (*Cyprinus carpio* L.) for resistance to dropsy: principal results and prospects. *Aquaculture,* 111**:** 7-20.

Kjøglum, S., Henryon, M., Aasmundstad, T. & Korsgaard, I. (2008). Selective breeding can increase resistance of Atlantic salmon to furunculosis, infectious salmon anaemia and infectious pancreatic necrosis. *Aquaculture Research,* 39**:** 498-505.

Krause, A., Neitz, S., Mägert, H.-J., Schulz, A., Forssmann, W.-G., Schulz-Knappe, P. & Adermann, K. (2000). LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. *FEBS letters,* 480**:** 147-150.

Kylsten, P., Samakovlis, C. & Hultmark, D. (1990). The cecropin locus in Drosophila; a compact gene cluster involved in the response to infection. *The EMBO journal,* 9**:** 217.

Lee, J.-Y., Boman, A., Sun, C., Andersson, M., Jörnvall, H., Mutt, V. & Boman, H.G. (1989). Antibacterial peptides from pig intestine: isolation of a mammalian cecropin. *Proceedings of the National Academy of Sciences,* 86**:** 9159-9162.

Lee, L.-H., Hui, C.-F., Chuang, C.-M. & Chen, J.-Y. (2013). Electrotransfer of the epinecidin-1 gene into skeletal muscle enhances the antibacterial and immunomodulatory functions of a marine fish, grouper (*Epinephelus coioides*). *Fish & shellfish immunology,* 35**:** 1359-1368.

Leeds, T., Silverstein, J., Weber, G., Vallejo, R., Palti, Y., Rexroad, C., Evenhuis, J., Hadidi, S., Welch, T. & Wiens, G. (2010). Response to selection for bacterial cold water disease resistance in rainbow trout. *Journal of Animal Science,* 88**:** 1936-1946.

Li, C., Wang, R., Su, B., Luo, Y., Terhune, J., Beck, B. & Peatman, E. (2013). Evasion of mucosal defenses during *Aeromonas hydrophila* infection of channel catfish (*Ictalurus punctatus*) skin. *Developmental & Comparative Immunology,* 39**:** 447-455.

Lin, C.-Y., Yang, P.-H., Kao, C.-L., Huang, H.-I. & Tsai, H.-J. (2010). Transgenic zebrafish eggs containing bactericidal peptide is a novel food supplement enhancing resistance to pathogenic infection of fish. *Fish & shellfish immunology,* 28**:** 419-427.

Ling, F., Luo, Q., Wang, J.-G., Wang, Y.-P., Wang, W.-B. & Gong, X.-N. (2009). Effects of the "all-fish" GH (growth hormone) transgene expression on resistance to *Ichthyophthirius multifiliis* infections in common carp, *Cyprinus carpio* L. *Aquaculture,* 292**:** 1-5.

Linn, F., Heidmann, I., Saedler, H. & Meyer, P. (1990). Epigenetic changes in the expression of the maize A1 gene in Petunia hybrida: role of numbers of integrated gene copies and state of methylation. *Molecular & general genetics: MGG,* 222**:** 329-336.

Liu, P., Wang, L., Wan, Z.Y., Ye, B.Q., Huang, S., Wong, S.-M. & Yue, G.H. (2016a). Mapping QTL for Resistance Against Viral Nervous Necrosis Disease in Asian Seabass. *Marine biotechnology,* 18**:** 107-116.

Liu, Z., Liu, S., Yao, J., Bao, L., Zhang, J., Li, Y., Jiang, C., Sun, L., Wang, R. & Zhang, Y. (2016b). The channel catfish genome sequence provides insights into the evolution of scale formation in teleosts. *Nature communications,* 7.

Lönnerdal, B. & Iyer, S. (1995). Lactoferrin: molecular structure and biological function. *Annual review of nutrition,* 15**:** 93-110.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., Dicarlo, J.E., Norville, J.E. & Church, G.M. (2013). RNA-guided human genome engineering via Cas9. *Science,* 339**:** 823-826.

Mao, Z., Bozzella, M., Seluanov, A. & Gorbunova, V. (2008). Comparison of nonhomologous end joining and homologous recombination in human cells. *DNA repair,* 7**:** 1765-1771.

Massault, C., Franch, R., Haley, C., De Koning, D., Bovenhuis, H., Pellizzari, C., Patarnello, T. & Bargelloni, L. (2011). Quantitative trait loci for resistance to fish pasteurellosis in gilthead sea bream (*Sparus aurata*). *Animal Genetics,* 42**:** 191-203.

Maynard, B.T., Taylor, R.S., Kube, P.D., Cook, M.T. & Elliott, N.G. (2016). Salmonid heterosis for resistance to amoebic gill disease (AGD). *Aquaculture,* 451**:** 106-112.

Meng, L., Wan, Y., Sun, Y., Zhang, Y., Wang, Z., Song, Y. & Wang, F. (2013). Generation of five human lactoferrin transgenic cloned goats using fibroblast cells and their methylation status of putative differential methylation regions of IGF2R and H19 imprinted genes. *PloS one,* 8**:** e77798.

Milani, A., Benedusi, M., Aquila, M. & Rispoli, G. (2009). Pore forming properties of cecropin-melittin hybrid peptide in a natural membrane. *Molecules,* 14**:** 5179-5188.

Miller, J.C., Holmes, M.C., Wang, J., Guschin, D.Y., Lee, Y.-L., Rupniewski, I., Beausejour, C.M., Waite, A.J., Wang, N.S. & Kim, K.A. (2007). An improved zinc-finger nuclease architecture for highly specific genome editing. *Nature biotechnology,* 25**:** 778-785.

Moen, T., Baranski, M., Sonesson, A.K. & Kjøglum, S. (2009). Confirmation and fine-mapping of a major QTL for resistance to infectious pancreatic necrosis in Atlantic salmon (*Salmo salar*): population-level associations between markers and trait. *BMC genomics,* 10**:** 1.

Moen, T., Sonesson, A.K., Hayes, B., Lien, S., Munck, H. & Meuwissen, T.H. (2007). Mapping of a quantitative trait locus for resistance against infectious salmon anaemia in Atlantic

salmon (*Salmo salar*): comparing survival analysis with analysis on affected/resistant data. *BMC genetics,* 8**:** 1.

Mohammed, H.H. (2015). Studies on the Epidemiology, Vaccination, Susceptibility, and Treatment of Columnaris Disease in Fishes. Auburn University.

Moore, A.J., Beazley, W.D., Bibby, M.C. & Devine, D.A. (1996). Antimicrobial activity of cecropins. *Journal of Antimicrobial Chemotherapy,* 37**:** 1077-1089.

Morita, T., Yoshizaki, G., Kobayashi, M., Watabe, S. & Takeuchi, T. (2004). Fish eggs as bioreactors: the production of bioactive luteinizing hormone in transgenic trout embryos. *Transgenic research,* 13**:** 551-557.

MSU (2012). Annual case summary report of the Aquatic Research and Diagnostic Laboratory. . *Mississippi State University, College of Veterinary Medicine, Thad Cochran, Stoneville, MS*.

Mussolino, C., Alzubi, J., Fine, E.J., Morbitzer, R., Cradick, T.J., Lahaye, T., Bao, G. & Cathomen, T. (2014). TALENs facilitate targeted genome editing in human cells with high specificity and low cytotoxicity. *Nucleic acids research,* 42**:** 6762-6773.

Na-Nakorn, U., Chantsawang, S. & Tarnchalanukit, W. (1995). Response to mass selection for disease resistance in walking catfish, *Clarias macrocephalus*. *Journal of Applied Aquaculture,* 4**:** 65-74.

Nakamura, S., Kato, A. & Kobayashi, K. (1990). Novel bifunctional lysozyme–dextran conjugate that acts on both Gram-negative and Gram-positive bacteria. *Agricultural and biological chemistry,* 54**:** 3057-3059.

Ødegård, J., Baranski, M., Gjerde, B. & Gjedrem, T. (2011). Methodology for genetic evaluation of disease resistance in aquaculture species: challenges and future prospects. *Aquaculture research,* 42**:** 103-114.

Ødegård, J., Olesen, I., Gjerde, B. & Klemetsdal, G. (2007). Positive genetic correlation between resistance to bacterial (furunculosis) and viral (infectious salmon anaemia) diseases in farmed Atlantic salmon (*Salmo salar*). *Aquaculture,* 271**:** 173-177.

Okamoto, N., Tayama, T., Kawanobe, M., Fujiki, N., Yasuda, Y. & Sano, T. (1993). Resistance of a rainbow trout strain to infectious pancreatic necrosis. *Aquaculture,* 117**:** 71-76.

Omata, Y., Satake, M., Maeda, R., Saito, A., Shimazaki, K., Uzuka, Y., Tanabe, S., Sarashina, T., Mikami, T. & Yamauchi, K. (2001). Reduction of the infectivity of *Toxoplasma gondii* and *Eimeria stiedai* sporozoites by treatment with bovine lactoferricin. *Journal of Veterinary Medical Science,* 63**:** 187-190.

Owens, L. (2012). Diseases. IN Lucas, J.S. & Southgate, P.C. (Eds.) *Aquaculture: Farming Aquatic Animals and Plants.* 2nd ed.: John Wiley & Sons.

Ozaki, A., Araki, K., Okamoto, H., Okauchi, M., Mushiake, K., Yoshida, K., Tsuzaki, T., Fuji, K., Sakamoto, T. & Okamoto, N. (2012). Progress of DNA marker-assisted breeding in maricultured finfish. *Bulletin of Fisheries Research Agency,* 35**:** 31-37.

Ozaki, A., Sakamoto, T., Khoo, S., Nakamura, K., Coimbra, M., Akutsu, T. & Okamoto, N. (2001). Quantitative trait loci (QTLs) associated with resistance/susceptibility to infectious pancreatic necrosis virus (IPNV) in rainbow trout (*Oncorhynchus mykiss*). *Molecular Genetics and Genomics,* 265**:** 23-31.

Padi, J. (2003). Genetic studies on reproduction, bacterial disease resistance and tolerance of adverse water quality in channel catfish, *Ictalurus punctatus*. PhD Dissertation, Auburn University, AL.

Pan, C.-Y., Chen, J.-Y., Cheng, Y.-S.E., Chen, C.-Y., Ni, I.-H., Sheen, J.-F., Pan, Y.-L. & Kuo, C.-M. (2007). Gene expression and localization of the epinecidin-1 antimicrobial peptide in the grouper (*Epinephelus coioides*), and its role in protecting fish against pathogenic infection. *DNA and cell biology,* 26**:** 403-413.

Pan, C.-Y., Chen, J.-Y., Lin, T.-L. & Lin, C.-H. (2009). In vitro activities of three synthetic peptides derived from epinecidin-1 and an anti-lipopolysaccharide factor against *Propionibacterium acnes*, *Candida albicans*, and *Trichomonas vaginalis*. *Peptides,* 30**:** 1058-1068.

Pan, C.-Y., Huang, T.-C., Wang, Y.-D., Yeh, Y.-C., Hui, C.-F. & Chen, J.-Y. (2012). Oral administration of recombinant epinecidin-1 protected grouper (*Epinephelus coioides*) and zebrafish (*Danio rerio*) from *Vibrio vulnificus* infection and enhanced immune-related gene expressions. *Fish & shellfish immunology,* 32**:** 947-957.

Pan, C.-Y., Peng, K.-C., Lin, C.-H. & Chen, J.-Y. (2011). Transgenic expression of tilapia hepcidin 1-5 and shrimp chelonianin in zebrafish and their resistance to bacterial pathogens. *Fish & shellfish immunology,* 31**:** 275-285.

Park, S.S., Shin, S.W., Park, D.-S., Oh, H.W., Boo, K.S. & Park, H.-Y. (1997). Protein purification and cDNA cloning of a cecropin-like peptide from the larvae of fall webworm (*Hyphantria cunea*). *Insect biochemistry and molecular biology,* 27**:** 711-720.

Pattanayak, V., Ramirez, C.L., Joung, J.K. & Liu, D.R. (2011). Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. *Nature methods,* 8**:** 765-770.

Pavlovic, J., Arzet, H.A., Hefti, H.P., Frese, M., Rost, D., Ernst, B., Kolb, E., Staeheli, P. & Haller, O. (1995). Enhanced virus resistance of transgenic mice expressing the human MxA protein. *Journal of virology,* 69**:** 4506-4510.

Peatman, E., Baoprasertkul, P., Terhune, J., Xu, P., Nandi, S., Kucuktas, H., Li, P., Wang, S., Somridhivej, B. & Dunham, R. (2007). Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium. *Developmental & Comparative Immunology,* 31**:** 1183-1196.

Peatman, E., Terhune, J., Baoprasertkul, P., Xu, P., Nandi, S., Wang, S., Somridhivej, B., Kucuktas, H., Li, P. & Dunham, R. (2008). Microarray analysis of gene expression in the blue catfish liver reveals early activation of the MHC class I pathway after infection with *Edwardsiella ictaluri*. *Molecular immunology,* 45**:** 553-566.

Peng, K.-C., Pan, C.-Y., Chou, H.-N. & Chen, J.-Y. (2010). Using an improved Tol2 transposon system to produce transgenic zebrafish with epinecidin-1 which enhanced resistance to bacterial infection. *Fish & shellfish immunology,* 28**:** 905-917.

Pigeon, C., Ilyin, G., Courselaud, B., Leroyer, P., Turlin, B., Brissot, P. & Loréal, O. (2001). A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *Journal of biological chemistry,* 276**:** 7811-7819.

Plumb, J.A. & Hanson, L.A. (2011). *Health maintenance and principal microbial diseases of cultured fishes*: John Wiley & Sons.

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

Prarom, W. (1990). Effect of strain crossing of gunther's walking catfish (*Clarias macrocephalus*) on growth and disease resistance.

Price, D. (1985). Genetics of susceptibility and resistance to disease in fishes. *Journal of fish biology,* 26**:** 509-519.

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

Qin, Z., Li, Y., Su, B., Cheng, Q., Ye, Z., Perera, D.A., Fobes, M., Shang, M. & Dunham, R.A. (2016). Editing of the Luteinizing Hormone Gene to Sterilize Channel Catfish, *Ictalurus punctatus*, Using a Modified Zinc Finger Nuclease Technology with Electroporation. *Marine biotechnology,* 18**:** 255-263.

Robertson, G., Garrick, D., Wu, W., Kearns, M., Martin, D. & Whitelaw, E. (1995). Position-dependent variegation of globin transgene expression in mice. *Proceedings of the National Academy of Sciences,* 92**:** 5371-5375.

Rodríguez-Ramilo, S.T., De La Herrán, R., Ruiz-Rejón, C., Hermida, M., Fernández, C., Pereiro, P., Figueras, A., Bouza, C., Toro, M.A. & Martínez, P. (2014). Identification of quantitative trait loci associated with resistance to viral haemorrhagic septicaemia (VHS) in turbot (*Scophthalmus maximus*): a comparison between bacterium, parasite and virus diseases. *Marine biotechnology,* 16**:** 265-276.

Rodríguez-Ramilo, S.T., Toro, M.A., Bouza, C., Hermida, M., Pardo, B.G., Cabaleiro, S., Martínez, P. & Fernández, J. (2011). QTL detection for *Aeromonas salmonicida* resistance related traits in turbot (*Scophthalmus maximus*). *BMC genomics,* 12**:** 1.

Røed, K., Fevolden, S.-E. & Fjalestad, K. (2002). Disease resistance and immune characteristics in rainbow trout (*Oncorhynchus mykiss*) selected for lysozyme activity. *Aquaculture,* 209**:** 91-101.

Sabl, J.F. & Henikoff, S. (1996). Copy number and orientation determine the susceptibility of a gene to silencing by nearby heterochromatin in Drosophila. *Genetics,* 142**:** 447-458.

Sander, J.D., Cade, L., Khayter, C., Reyon, D., Peterson, R.T., Joung, J.K. & Yeh, J.-R.J. (2011). Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nature biotechnology,* 29**:** 697-698.

Sarmasik, A., Warr, G. & Chen, T.T. (2002). Production of transgenic medaka with increased resistance to bacterial pathogens. *Marine biotechnology,* 4**:** 310-322.

Schwartz, T., Kohnen, W., Jansen, B. & Obst, U. (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS microbiology ecology,* 43**:** 325-335.

Shike, H., Shimizu, C., Lauth, X. & Burns, J.C. (2004). Organization and expression analysis of the zebrafish hepcidin gene, an antimicrobial peptide gene conserved among vertebrates. *Developmental & Comparative Immunology,* 28**:** 747-754.

Shimada, T., Park, B.G., Wolf, A.J., Brikos, C., Goodridge, H.S., Becker, C.A., Reyes, C.N., Miao, E.A., Aderem, A. & Götz, F. (2010). *Staphylococcus aureus* evades lysozyme-based peptidoglycan digestion that links phagocytosis, inflammasome activation, and IL-1β secretion. *Cell host & microbe,* 7**:** 38-49.

Silverstein, P., Bosworth, B. & Gaunt, P. (2008). Differential susceptibility of blue catfish, *Ictalurus furcatus* (Valenciennes), channel catfish, *I. punctatus* (Rafinesque), and blue x channel catfish hybrids to channel catfish virus. *Journal of fish diseases,* 31**:** 77.

Smitherman, R. & Dunham, R. (1985). Genetics and breeding. IN Tucker, C.S. (Ed.) *Channel catfish culture.* Amsterdam: Elsevier Scientific Publishing.

Sommerset, I., Krossøy, B., Biering, E. & Frost, P. (2014). Vaccines for fish in aquaculture. *Expert review of vaccines.*

Soto, E., Kidd, S., Gaunt, P. & Endris, R. (2013). Efficacy of florfenicol for control of mortality associated with *Francisella noatunensis* subsp. orientalis in Nile tilapia, *Oreochromis niloticus* (L.). *Journal of fish diseases,* 36**:** 411-418.

Stciner, H., Hultmark, D., Engstrom, A., Bennich, H. & Barman, H. (1981). Séquence and specificity of two antibacterial proteins involved in insect immunity. *Nature,* 292**:** 246-8.

Stuart, G.W., Mcmurray, J.V. & Westerfield, M. (1988). Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. *Development,* 103**:** 403-412.

Su, B. (2012). Reproductive confinement of common carp, *Cyprinus carpio*, and channel catfish, *Ictalurus punctatus*, via transgenic sterilization. Auburn University.

Su, J., Yang, C., Zhu, Z., Wang, Y., Jang, S. & Liao, L. (2009). Enhanced grass carp reovirus resistance of Mx-transgenic rare minnow (*Gobiocypris rarus*). *Fish & shellfish immunology,* 26**:** 828-835.

Sun, F., Peatman, E., Li, C., Liu, S., Jiang, Y., Zhou, Z. & Liu, Z. (2012). Transcriptomic signatures of attachment, NF-κB suppression and IFN stimulation in the catfish gill following columnaris bacterial infection. *Developmental & Comparative Immunology,* 38**:** 169-180.

Tang, J., Scarth, R. & Fristensky, B. (2003). Effects of genomic position and copy number of Acyl-ACP thioesterase transgenes on the level of the target fatty acids in *Brassica napus* L. *Molecular Breeding,* 12**:** 71-81.

Tao, Y., Zhao, D.-M. & Wen, Y. (2014). Expression, purification and antibacterial activity of the channel catfish hepcidin mature peptide. *Protein expression and purification,* 94**:** 73-78.

Terns, M.P. & Terns, R.M. (2011). CRISPR-based adaptive immune systems. *Current opinion in microbiology,* 14**:** 321-327.

Tewari, R., Michard-Vanhee, C., Perrot, E. & Chourrout, D. (1992). Mendelian transmission, structure and expression of transgenes following their injection into the cytoplasm of trout eggs. *Transgenic Research,* 1**:** 250-260.

Thongda, W., Li, C., Luo, Y., Beck, B.H. & Peatman, E. (2014). L-rhamnose-binding lectins (RBLs) in channel catfish, *Ictalurus punctatus*: Characterization and expression profiling in mucosal tissues. *Developmental & Comparative Immunology,* 44**:** 320-331.

Tryselius, Y., Samakovlis, C., Kimbrell, D.A. & Hultmark, D. (1992). CecC, a cecropin gene expressed during metamorphosis in Drosophila pupae. *European journal of biochemistry,* 204**:** 395-399.

Tucker, C. (2012). Channel Catfish. IN Lucas, J.S. & Southgate, P.C. (Eds.) *Aquaculture: Farming Aquatic Animals and Plants.* 2nd ed.: John Wiley & Sons.

Turchany, J.M., Aley, S.B. & Gillin, F.D. (1995). Giardicidal activity of lactoferrin and N-terminal peptides. *Infection and immunity,* 63**:** 4550-4552.

Urnov, F.D., Miller, J.C., Lee, Y.-L., Beausejour, C.M., Rock, J.M., Augustus, S., Jamieson, A.C., Porteus, M.H., Gregory, P.D. & Holmes, M.C. (2005). Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature,* 435**:** 646-651.

Van Der Strate, B., Beljaars, L., Molema, G., Harmsen, M. & Meijer, D. (2001). Antiviral activities of lactoferrin. *Antiviral research,* 52**:** 225-239.

Varshney, G.K., Pei, W., Lafave, M.C., Idol, J., Xu, L., Gallardo, V., Carrington, B., Bishop, K., Jones, M. & Li, M. (2015). High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. *Genome research,* 25**:** 1030-1042.

Veres, A., Gosis, B.S., Ding, Q., Collins, R., Ragavendran, A., Brand, H., Erdin, S., Cowan, C.A., Talkowski, M.E. & Musunuru, K. (2014). Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing. *Cell stem cell,* 15**:** 27-30.

Vike, S., Nylund, S. & Nylund, A. (2009). ISA virus in Chile: evidence of vertical transmission. *Archives of virology,* 154**:** 1-8.

Wachinger, M., Kleinschmidt, A., Winder, D., Von Pechmann, N., Ludvigsen, A., Neumann, M., Holle, R., Salmons, B., Erfle, V. & Brack-Werner, R. (1998). Antimicrobial peptides melittin and cecropin inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression. *Journal of General Virology,* 79**:** 731-740.

Wang, R., Zhang, P., Gong, Z. & Hew, C.L. (1995). Expression of the antifreeze protein gene in transgenic goldfish (*Carassius auratus*) and its implication in cold adaptation. *Molecular Marine Biology and Biotechnology,* 4**:** 20-26.

Wang, W.-B., Wang, Y.-P., Hu, W., Li, A.-H., Cai, T.-Z., Zhu, Z.-Y. & Wang, J.-G. (2006). Effects of the "all-fish" growth hormone transgene expression on non-specific immune functions of common carp, *Cyprinus carpio* L. *Aquaculture,* 259**:** 81-87.

Wang, Y.-D., Kung, C.-W. & Chen, J.-Y. (2010). Antiviral activity by fish antimicrobial peptides of epinecidin-1 and hepcidin 1–5 against nervous necrosis virus in medaka. *Peptides,* 31**:** 1026-1033.

Wang, Y.-D., Peng, K.-C., Wu, J.-L. & Chen, J.-Y. (2014). Transgenic expression of salmon delta-5 and delta-6 desaturase in zebrafish muscle inhibits the growth of *Vibrio alginolyticus* and affects fish immunomodulatory activity. *Fish & shellfish immunology,* 39**:** 223-230.

Weifeng, M., Yaping, W., Wenbo, W., Bo, W., Jianxin, F. & Zuoyan, Z. (2004). Enhanced resistance to *Aeromonas hydrophila* infection and enhanced phagocytic activities in human lactoferrin-transgenic grass carp (*Ctenopharyngodon idellus*). *Aquaculture,* 242**:** 93-103.

Wetten, M., Aasmundstad, T., Kjøglum, S. & Storset, A. (2007). Genetic analysis of resistance to infectious pancreatic necrosis in Atlantic salmon (*Salmo salar* L.). *Aquaculture,* 272**:** 111-117.

Wolters, W., Waldbieser, G., Bosworth, B., Silverstein, J. & Robinson, E. (2002). Genetically distinct strain of channel catfish designated NWAC103, with improved growth performance. Google Patents.

Wolters, W.R. & Johnson, M.R. (1994). Enteric septicemia resistance in blue catfish and three channel catfish strains. *Journal of Aquatic Animal Health,* 6**:** 329-334.

Wolters, W.R. & Johnson, M.R. (1995). Analysis of a diallel cross to estimate effects of crossing on resistance to enteric septicemia in channel catfish, *Ictalurus punctatus*. *Aquaculture,* 137**:** 263-269.

Wolters, W.R., Wise, D.J. & Klesius, P.H. (1996). Survival and antibody response of channel catfish, blue catfish, and channel catfish female× blue catfish male hybrids after exposure to *Edwardsiella ictaluri*. *Journal of Aquatic Animal Health,* 8**:** 249-254.

Xu, C., Wu, G., Zohar, Y. & Du, S.-J. (2003). Analysis of myostatin gene structure, expression and function in zebrafish. *Journal of Experimental Biology,* 206**:** 4067-4079.

Xu, D.-H., Klesius, P.H., Peatman, E. & Liu, Z. (2011). Susceptibility of channel catfish, blue catfish and channel× blue catfish hybrid to *Ichthyophthirius multifiliis*. *Aquaculture,* 311**:** 25-30.

Yamauchi, K., Tomita, M., Giehl, T. & Ellison, R.R. (1993). Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. *Infection and immunity,* 61**:** 719-728.

Yazawa, R., Hirono, I. & Aoki, T. (2006). Transgenic zebrafish expressing chicken lysozyme show resistance against bacterial diseases. *Transgenic research,* 15**:** 385-391.

Yoshizaki, G., Kiron, V., Satoh, S. & Takeuchi, T. (2007). Expression of masu salmon Δ5-desaturase-like gene elevated EPA and DHA biosynthesis in zebrafish. *Marine biotechnology,* 9**:** 92-100.

Zapata, A., Torroba, M., Varas, A. & Jimenez, A. (1996). Immunity in fish larvae. *Developments in biological standardization,* 90**:** 23-32.

Zhong, J., Wang, Y. & Zhu, Z. (2002). Introduction of the human lactoferrin gene into grass carp (*Ctenopharyngodon idellus*) to increase resistance against GCH virus. *Aquaculture,* 214**:** 93-101.

Zhou, T., Liu, S., Geng, X., Jin, Y., Jiang, C., Bao, L., Yao, J., Zhang, Y., Zhang, J. & Sun, L. (2016). GWAS analysis of QTL for enteric septicemia of catfish and their involved genes suggest evolutionary conservation of a molecular mechanism of disease resistance. *Molecular Genetics and Genomics***:** 1-12.

Chapter 2: Microinjection of CRISPR/Cas9 protein into channel catfish, *Ictalurus punctatus*, embryos for gene editing

Abstract

The complete genome of channel catfish has been sequenced, leading to greater opportunity to study gene function in channel catfish (*Ictalurus punctatus*). Gene knockout has been used to study gene functions *in vivo*. Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9) system is a powerful tool used to edit genomic DNA sequences. The traditional approach has been to introduce CRISPR/Cas9 mRNA into the single cell embryos through microinjection. Microinjection can be a slow an inefficient process in catfish. Here, a detailed protocol for microinjection of channel catfish embryos with CRISPR/Cas9 protein is described. Briefly, eggs and sperm were collected then artificial fertilization performed. Fertilized eggs were transferred to a petri dish containing Holtfreter's solution. A microinjection needle was loaded with the injection solution then connected to a microinjector. Injection volume was determined with a hemocytometer by injecting into a drop of mineral oil. Once the needle was adjusted to inject the required volume, fertilized eggs were microinjected. The needle is introduced into the yolk and injection material is expelled. Embryos were then incubated in Holtfreter's solution until hatch. To demonstrate the efficiency of this approach, toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene were targeted for gene knockout in channel catfish. Indels were confirmed by DNA sequencing. Dramatic changes in the predicted protein sequence included frameshift and truncated protein due to premature stop codon.

1. Introduction

Microinjection is a common laboratory technique used to deliver a small amount of a substance such as DNA, RNA, protein and other macromolecules into cells or embryos through a glass capillary (Hammer et al., 1985). Microinjection is performed using a special equipment set up including microinjector, micromanipulator and a microscope (Komarova et al., 2011). The technique has been used by many researchers to genetically modify organisms through generation of transgenics, gene knockouts, gene therapy and to understand the dynamics of intracellular components (Du et al., 1992, Davis et al., 2000, Jao et al., 2013).

Channel catfish (*Ictalurus punctatus*) is the most popular catfish species in the United States. There is an increasing need to study functional genomics in channel catfish especially now that the complete genome of channel catfish has been sequenced (Liu et al., 2016). Understanding gene function would not only enrich the research that is being conducted on catfish, but could also lead to more effective genetic improvement programs to enhance the catfish industry. Once critical genes for a given trait of interest are identified, they can be used to genetically improve catfish production through selection for these genes, suppressing them or transferring them through transgenesis or combinations of these options. Combining the best genes for different commercially beneficial traits from different species in one fish would greatly improve productivity and profitability of fish production operations (Dunham, 2011).

Gene knockout is a direct method to study gene functions *in vivo*. Mutations at the DNA level will be inherited to future generations which will facilitate the study of their effects in different generations. Different genome editing tools have been developed including zinc finger nucleases (ZFNs) (Doyon et al., 2008), transcription activator-like effector nucleases (TALENs)

(Miller et al., 2011) and clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system (Ran et al., 2013).

CRISPR/Cas9 system is a powerful, efficient tool that has been used to edit genomic DNA sequences including gene knockout in fish through RNA-guided site-specific DNA cleavage (Jao et al., 2013). The system consists of a guide RNA (gRNA), which determines the targeted sequence in the genome and a DNA endonuclease enzyme, Cas9. CRISPR/Cas9 system can be designed to target any sequence in the genome with several advantages over ZFNs and TALENs including (1) lower cost, (2) easier engineering, (3) more specific binding of guide RNA to the target sequence reducing the off-target mutations (Hruscha et al., 2013), (4) multiple sequences can be targeted with different gRNA at the same time (Cong et al., 2013), (5) high mutagenesis rate in genes that could not be mutated by TALENs (Cradick et al., 2013) and (6) improved germline transmission rate of mutations up to 6-fold when compared to ZFNs and TALENs (Varshney et al., 2015).

The main alternative method for microinjection is the electroporation in which electric impulses are applied to embryos or cells to increase the membrane permeability and the uptake of biological molecules (Neumann et al., 1982, Powers et al., 1991). Several transgenic fish were generated using electroporation such as medaka (*Oryzias latipes*) (Inoue et al., 1990), zebrafish (*Danio rerio*) (Buono and Linser, 1991), chinook salmon (*Oncorhynchus tshawytscha*) (Sin et al., 1993), channel catfish (Dunham et al., 2002), sea bream (*Sparus sarba*) (Lu et al., 2002) and common carp (*Cyprinus carpio*)(Cheng et al., 2014).

Electroporation has been used to deliver plasmid DNA, RNA and Cas9 protein for gene knockout. In mammalian cells, the delivery of Cas9/gRNA plasmid DNA, Cas9 mRNA/gRNA

and Cas9 protein/gRNA complexes using electroporation was compared (Liang et al., 2015). In the ascidian chordate (*Ciona intestinalis*), TALENs expressing constructs were electroporated into fertilized eggs to induce knockout of multiple genes (Treen et al., 2014). Oin et al. (2016) electroporated ZFNs expressing plasmid constructs to knock out luteinizing hormone in channel catfish. However, once introduced into the cell, plasmid constructs will need to be transcribed to RNA and translated to functional proteins before they can target the desired DNA sequence. Simultaneously, the single cell embryo is developing, which makes mutation of the genome less likely at the one cell stage using this technique.

As a means for introducing genome editing tools into cells, microinjection has several advantages over electroporation. (1) Genome editing molecules can be reliably introduced into cells or embryos through microinjection. (2) Less injection material is needed. (3) It is easy to determine the amounts of material injected. (4) Higher mutation rates with low mosaicism in the founder fish can be achieved which would improve germline transmission of mutations to F_1. (5) Fewer founder fish need to be analyzed to produce the first generation due to the higher mutation rate. In electroporation, more founder fish need to be analyzed which will increase the costs. However, the survival of microinjected embryos is lower than electroporated ones, but this can be overcome by injecting more embryos (Dunham and Winn, 2014).

Regular microinjection of embryos is technically demanding and time consuming, however, a rapid efficient CRISPR/Cas9 protein microinjection protocol is presented for channel catfish embryos. This protocol requires less time and expertise since CRISPR/Cas9 protein are injected into the yolk of one cell embryos. Hundreds of fertilized eggs can be injected in 1 hour (approximately the time needed for the first cell division to occur). Two disease susceptibility-

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related genes, toll/interleukin 1 receptor domain-containing adapter molecule (TICAM1) gene and rhamnose binding lectin (RBL) gene, were knocked out in channel catfish, using CRISPR/Cas9 protein system to validate the protocol for CRISPR gene editing.

2. Materials and methods

This experiment was conducted at the Fish Genetics Research Unit, E. W. Shell Fisheries Research Center, Auburn University, AL. All experimental protocols used in this experiment were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC) before the experiment was initiated. A list of the equipment and supplies used in this experiment can be found in Table 1. The following are the steps and procedures for preparation and microinjection of channel catfish one-cell embryos as illustrated in Figure 1. All microinjection procedures have been photographed and filmed and the video can be accessed from the URL: https://youtu.be/oOYwp23utsE

2.1. Brood stock selection and spawning

2.1.1. Choose healthy channel catfish brood stock from the strain or genetic line that you would like to gene edit. Channel catfish males and females should exhibit good secondary sex characteristics.

2.1.2. Select males that have a broad muscular head that is wider than the rest of their bodies with well-developed genital papilla. Dark color and scarring and wounds from territorial fighting are also signs of sexual readiness. Select females that have heads that are narrower than the rest of their bodies, and have soft, palpable, well-rounded abdomen. For accuracy and to avoid stressing the fish during initial handling, stop feeding the females 2-3 days before examining their readiness for spawning.

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Table 1. Name, type, company, catalog number (if applicable) of materials and equipment used to validate the microinjection protocol for gene knockout in channel catfish, *Ictalurus punctatus*.

Name of Material/ Equipment	Type	Company	Catalog Number	Comments/Description
Reproboost® implant	Hormone	Center of Marine Biotechnology		Luteinizing hormone releasing hormone analog (LHRHa) for artificial spawning
TRICAINE-S	Anesthesia	Western Chemical. Inc.		For sedation of brood stock fish during hormone injection and egg stripping.
Phenol red	Reagent	Sigma-Aldrich	P0290	0.5%, sterile filtered
Stereo microscope	Equipment	Olympus	213709	For visualizing the eggs during microinjection
Microinjector	Equipment	ASI-Applied Scientific Insturmentation	Model MPPI-3	For the delivery of the injection material into the embryos
Micromanipulator	Equipment	ASI-Applied Scientific Insturmentation	Model MM33	For holding and controlling the movement of the injection needle.
Eppendorf Microloader	Tool	Eppendorf	5242956.003	For loading injection solution into microinjection needles.
Vertical needle puller	Equipment	David Kopf Instruments	Model 720	For pulling microinjection needles

Table 1 *Continued*.

Name of Material/ Equipment	Type	Company	Catalog Number	Comments/Description
Vertical needle puller	Equipment	David Kopf Instruments	Model 720	For pulling microinjection needles
Borosilicate glass capillaries	Tool	Fisher Scientific		1 mm outer diameter (OD), for making microinjection needles.
Petri dish	Tool	VWR	25384-302	For holding the embryos during the microinjection.
Crisco®	Vegetable shortening	The J.M. Smucker Company		For coating spawning pans and petri dishes.
Holtfreter`s solution	Reagent	Home Made		59 mM NaCl, 0.67 mM KCl, 2.4 mM NaHCO$_3$, 0.76 mM CaCl$_2$, 1.67 mM MgSO$_4$ (Armstrong et al., 1989) to incubate the microinjected embryos till hatch.
Doxycycline hyclate USP (monohydrate)	Antibiotic	Letco Medical	690904	Added to Holtfreter's solution to 10 ppm to prevent bacterial infections.
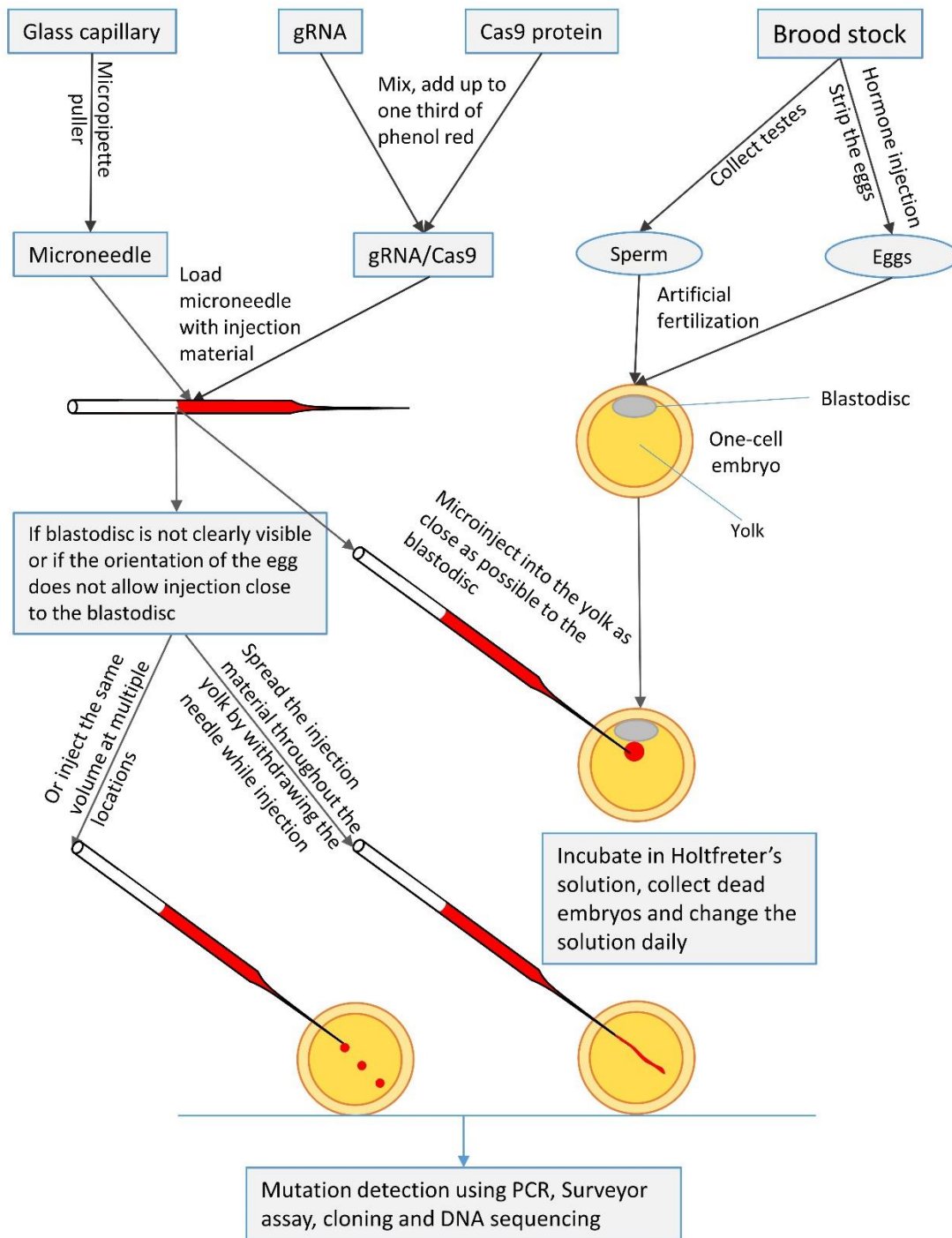
Figure 1 A summary of the microinjection procedures of one-cell channel catfish (*Ictalurus punctatus*) embryos with gRNA/Cas9 protein to knock out toll/interleukin 1 receptor domain-containing adapter molecule (TICAM1) gene and rhamnose binding lectin (RBL) gene.

2.1.3. Fish handling should be done quickly, but carefully. To reduce handling stress, all procedures that require handling of the females should be done while fish are held in spawning bags (32 mm size mesh).

2.1.4. Just before the hormone injection, obtain the body weight of the females.

2.1.5. Spawning bags with females inside can then be hung in a flow through tank with continuous water flow and adequate aeration.

2.1.6. Aseptically, load the implanter that has a 14-gauge needle with 85 µg/kg of luteinizing hormone releasing hormone analog (LHRHa) implant. Rinse the injection site (dorsal musculature) with 0.9% sterile saline solution before the injection. Insert the implanter at a 45-degree angle and release the implant. Withdraw the needle and swipe the injection site with a cotton previously soaked in 70% ethanol.

2.1.7. Place the spawning bag in the holding tank so that the fish is completely submerged in water 15-20 cm below the water surface. Adequate aeration (above 5 ppm dissolved oxygen) and good water quality are important for ovulation of high quality eggs.

2.1.8. Predict the ovulation time based on the water temperature using the degree-hour relationship (Phelps et al., 2007). Usually, the first check for eggs is done at about 36 hrs after hormone injection and then at 4 hrs interval.

2.1.9. To check for ovulation, gently lift the spawning bag above the water surface while you are looking for the eggs that are attached inside the spawning bag. When you see eggs (at least 10), this female is ovulating and ready for hand stripping.

2.2. Sperm preparation

2.2.1. Sperm can be prepared a few hours before the expected ovulation time.

2.2.2. Males are euthanized by a percussive blow to the head without anesthesia. Anesthesia might have a negative effect on the sperm motility.

2.2.3. Collect the testes in a small weighing pan, remove any tissue and wash them with 0.9% saline to remove blood, if necessary. Well-developed testes are white with many villiform projections.

2.2.4. Determine the weight of the testes.

2.2.5. Crush the testes and filter the sperm using a 100-micron mesh. Use 0.9% saline solution to wash the sperm from the mesh into a 50-mL tube. Saline solution can be added up to10 mL/gram of testes.

2.2.6. Determine the concentration of the sperm, check the motility and viability, however, for these procedures, this is not necessary if the testes are well developed.

2.2.7. Sperm can then be stored at 4°C and should be used within 24 hrs. after preparation.

2.3. Egg collection and fertilization

2.3.1. Place the female in the anesthetic solution containing 100 ppm buffered tricaine methane sulfonate (MS-222) with sodium bicarbonate until the fish is completely anesthetized.

2.3.2. Apply a very thin layer of vegetable shortening, e.g., Crisco, to a 20- cm diameter clean dry spawning pan.

2.3.3. Carefully, remove the fish from the spawning bag and dip it in 0.9% saline to wash off the MS-222. Dry the fish off completely using a clean towel avoiding the removal of the mucus layer on the fish body surface.

2.3.4. Apply vegetable shortening to the area around the vent including the pelvic fins to prevent attachment of the eggs during hand stripping.

2.3.5. Hand strip the eggs into the greased spawning pan by applying gentle pressure to the ovaries. Good eggs should flow freely, be golden yellow in color and have minimal or no clumps or blood clots. Avoid contact between eggs and water before fertilization as water can stimulate the micropyle closure.

2.3.6. To fertilize the eggs for microinjection, transfer 200-300 eggs to a greased spawning pan. Add 1-2 mL of the sperm solution to the eggs and mix gently.

2.3.7. Add freshwater to the eggs to activate the sperm and eggs. Enough water should be added to slightly cover the eggs. Gently swirl the eggs for 30 seconds. Fertilization should occur in 1-2 min. It is important to fertilize the eggs in a single layer. Catfish eggs adhere to each other making it difficult to microinject multiple layers of embryos.

2.3.8. Add more freshwater to the fertilized eggs and allow the eggs to harden for 10-15 min.

2.3.9. The remaining unfertilized eggs can be covered by a wet towel to prevent drying and can be fertilized over a few hours. Fertilization can be done in a staggered manner e.g. one batch of 200-300 eggs can be fertilized every 30-60 minute to ensure the continuous supply of embryos at the one cell stage for microinjection and control non-injected embryos. Avoid contact between the wet towel and the eggs. In this protocol, eggs fertilized within 2 – 3 hours after stripping were efficiently microinjected and seemed to have similar success as eggs fertilized immediately after stripping.

2.4. Needle pulling and loading

2.4.1. Pull a 1.0 mm OD borosilicate glass capillary into 2 needles (Figure 2). Store the needles in a paper box with a piece of sponge in which several incisions have been made. To avoid breaking the needle, the diameter of the sponge should be smaller than the length of the needle stem.

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2.4.2. The needle tip can then be opened by breaking a small piece of the needle finest region using sharp object. Alternatively, the needle can be opened by pinching off the thinnest region of the tip with a pair of forceps under the microscope.

2.4.3. Prepare the injection solution by mixing gRNA(s) with Cas9 protein. Other types of RNA or DNA can also be injected with the same procedure. In this protocol, gRNAs were designed to target two of the channel catfish immune related genes, toll/interleukin 1 receptor domain-containing adapter molecule (TICAM1) gene and rhamnose binding lectin (RBL) gene. The genomic targets for gRNAs were 5' GGA GGT GAA GCA CGT CGA GGA 3' for TICAM 1 gene (Figure 3) and 5' GGA CTT TGA GTC GGA GAA GTG G 3' for exon 1 of RBL gene with protospacer adjacent motif (PAM) sequence underlined (Figure 5). Phenol red can be added to color the gRNA/Cas9 protein mix with the phenol red constituting up to one third of the total volume. The mixture is then incubated for 10 minutes on ice before use.

2.4.4. With a microloader, load 5-10 µl of the gRNA/Cas9 mixture into the injection needle by inserting the microloader into the needle stem and expelling the mixture slowly while retracting the microloader tip (Figure 2). Avoid trapping air bubbles inside.

2.4.5. Attach the needle to the micropipette holder and ensure a tight connection, and then attach the holder to the micromanipulator. Ensure free stable movement. Apply pressure for microinjection by opening the nitrogen cylinder and adjusting the pressure regulator.

2.4.6. The volume of injection can be affected by the pressure, needle diameter and duration of injection. To determine the volume you wish to inject, inject into a drop of mineral oil placed on a hemocytometer. If needed, the pressure and needle diameter can be adjusted

to increase or decrease the volume of the injection. Inject multiple times in the mineral oil to ensure consistent volume.

2.5. Microinjection of catfish embryos

2.5.1. Apply a very thin layer of vegetable shortening to a 100-mm clean petri dish.

2.5.2. Transfer 50-100 eggs from the fertilization pan to the petri dish and cover them with Holtfreter's solution (Table 1). The eggs should be aligned against each other in a single layer. This alignment should hold the eggs in place during the microinjection process.

2.5.3. Place the petri dish with the eggs on the stage of the microscope and hold it with one hand. Lower the needle with the other hand (Figure 2) until it pierces the chorion and the yolk in a single smooth movement. The tip of the needle should be as close as possible to the blastodisc before delivering the injection material. Depress the pedal to deliver the injection material into the yolk. If the blastodisc is not clearly visible, then the injection material can be spread into different locations in the yolk. To do that, the needle is inserted to the far end of the yolk, then depressing the pedal and withdrawing the needle are done simultaneously to spread the injection material at different locations of the yolk. Fifty nanoliters can be injected into the yolk without any problems.

2.5.4. Retract the needle smoothly as not to damage the egg. Then move the petri dish to inject another egg with the same procedure. Avoid movement of the petri dish during the injection process as this may damage the egg or break the needle. Remove eggs that are ruptured or damaged due to microinjection. Injection can be started at 15-20 minutes after fertilization and continue as long as the embryos are still in the one cell stage (until about 90 minutes after fertilization).

2.5.5. Injected embryos are placed back in Holtfreter's solution with 10 ppm doxycycline and continuous aeration for 6-7 days until hatch. The solution is changed daily and dead embryos are removed.

3. Results

To demonstrate the efficiency of the microinjection protocol, gRNAs designed to target the channel catfish toll/interleukin 1 receptor domain-containing adapter molecule (TICAM1) gene and rhamnose binding lectin (RBL) gene were microinjected.

3.1. TICAM 1

DNA sequencing revealed the indel mutations induced in TICAM 1 gene. Out of 24 random individuals analyzed, 19 were mutated (79.2%). Examples of mutations are illustrated in Figure 3 and Figure 4. Effects of indel mutations in TICAM1 gene coding sequence of channel catfish on predicted protein length and sequence compared to the wild type sequence are illustrated in Table 2. Mutations resulted in removal of a few to several amino acids from the protein, changing the downstream reading frame and premature termination of translation.

In most cases, more than 80% of the protein sequence was truncated due to a premature stop codon. In these cases, the predicted polypeptide length ranged from 91 to 106 amino acid residues (wild type TICAM 1 has 520 amino acid residues). Mutations with extremely truncated protein are expected to be nonsense producing a non-functional protein. In the case of the 87 bp deletion mutation (Table 2, Figure 3), 29 amino acids were removed from the protein without changing the reading frame which may or may not result in loss of function depending on which domain of the protein is affected.

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3.2. RBL

DNA sequencing confirmed the presence of indel mutations (Figure 5). Out of 40 individuals analyzed, 35 were mutated (87.5%). Deletions ranged from 5 bp to 183 bp while up to 20 bp were inserted. Interestingly, the 183 bp deletions completely removed intron 1, exon 2 and 19 bp from intron 2. Theoretically, this should influence the splicing of the RBL gene since the splice sites have been mutated.

Indel mutations had variable effects on the predicted protein sequence when compared to the wild type protein sequence (308 amino acid residues) (Table 3). More than 70% of indels resulted in truncated protein that is 10% or less than the length of the wild type protein. In some indels (mutation number 4, 5, and 6), the predicted polypeptide was less than 10 amino acids. Only 2 cases (number 2 and 3) resulted in deletion of 2 and 4 amino acids without changing the reading frame which may or may not affect the protein function.
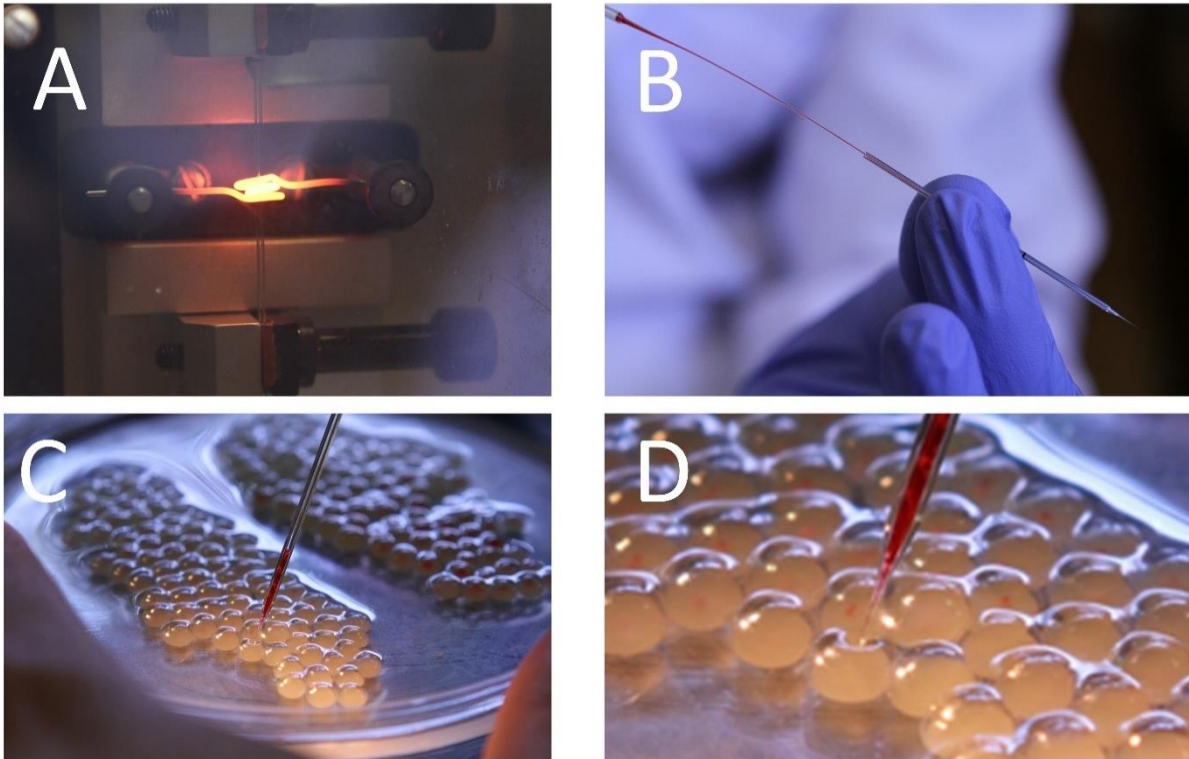
Figure 2. Microneedle pulling, loading for microinjection of one-cell channel catfish, *Ictalurus punctatus*, embryos. A: Needle pulling with a vertical needle puller. Needle structure and diameter are affected by the heat of the heater filament and the solenoid force. B: Microneedle loading with the CRISPR/Cas9 mix using microloader tip. C: Arrangement of channel catfish embryos in a 100-mm petri dish containing Holtfreter's solution. Microneedle is lowered until it touches and pierces the egg. D: Magnified section of panel C showing the process of piercing the egg. Note the needle tip is pushing the chorionic membrane inward but has not penetrated it yet. Injected embryos have the red injection material inside.
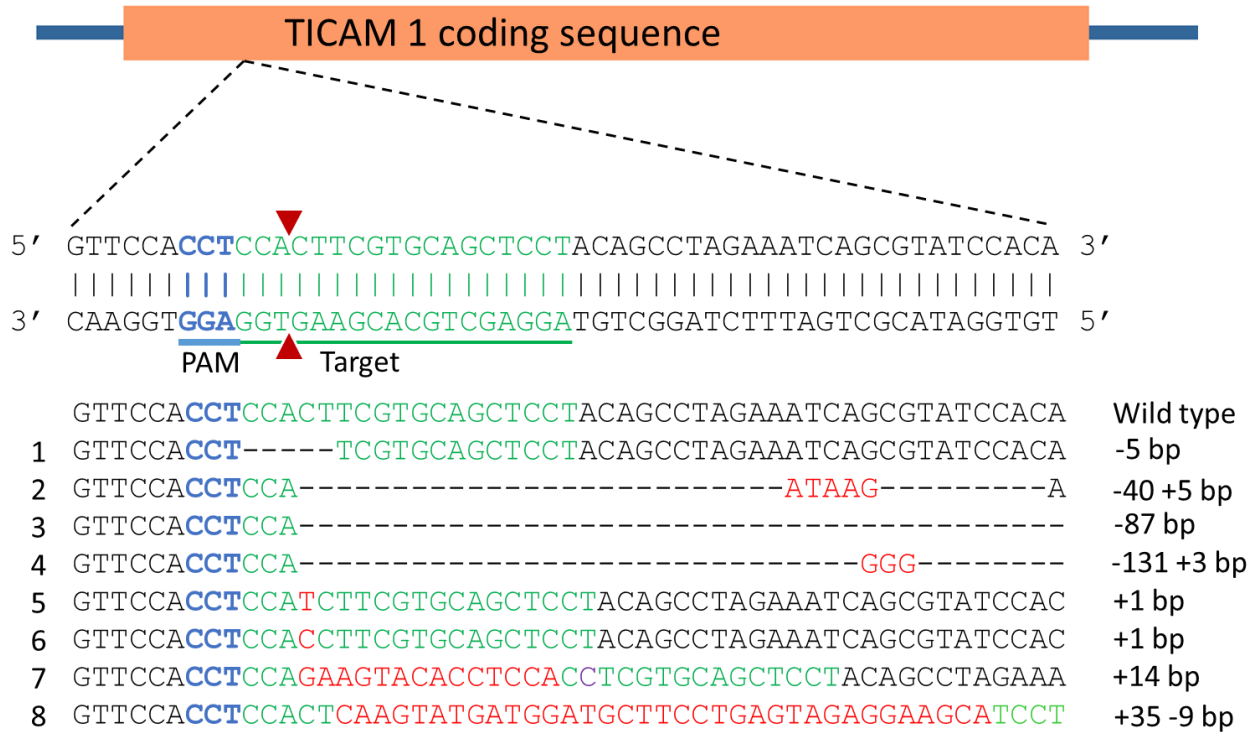
Figure 3. CRISPR/Cas9 induced mutations in toll/interleukin 1 receptor domain-containing adapter molecule (TICAM1) gene coding sequence of channel catfish, *Ictalurus punctatus*. Blue sequence represents the protospacer adjacent motif (PAM) while the green sequence represents the target for gRNA. Double strand break induced by Cas9 protein was expected to occur at the site of the 2 red triangles. Mutations are assigned numbers 1 through 8. Deletion mutations are represented by a dashed line in which each dash corresponds to a nucleotide that has been deleted. Red sequences are insertions while purple represents substitution mutation.
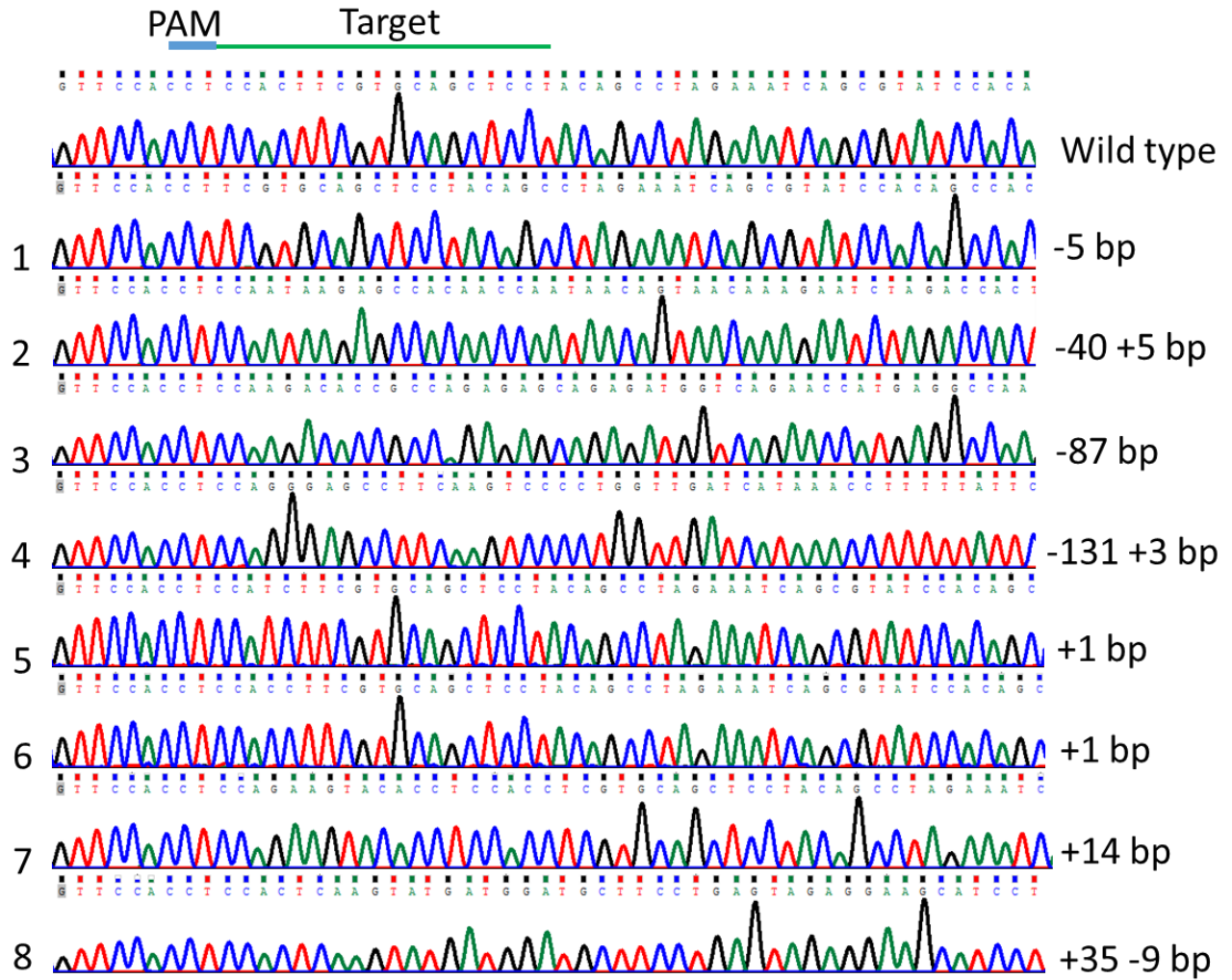
Figure 4. Indel mutations in the channel catfish, *Ictalurus punctatus*, toll/interleukin 1 receptor domain-containing adapter molecule (TICAM1) gene coding sequence induced by microinjection of gRNA/Cas9 protein into channel catfish one-cell embryos as revealed by DNA sequencing chromatogram.

Table 2. Effects of indel mutations in the toll/interleukin 1 receptor domain-containing adapter molecule (TICAM1) gene coding sequence of channel catfish, *Ictalurus punctatus*, on predicted protein length (amino acids) and sequence compared to the wild type sequence (WT).

Mutation	Insertion	Deletion	Net change (Δ)	Protein length (amino acid)	Frameshift changed	Premature stop codon	Predicted protein sequence
WT	0	0	0	520	No	No	MAEETELVDEKKPTELCVNRNTVVNSPLER SISGSRSTENISGEHTAAECLIMGASLQPRRE STEAFKVQKEPPKRDDSYPSSLRSTSTSCSSY SLEISVSTATTNNSNKESRPLPLKTPPESRDG QNHEAKPSSPLVDHKPFYSVTKSYKSSDPTP LQERAQLEKFKFRQYPDKHDTSEIVTPKSPNI ESGLEKTFLSIPSGGGKVGAQPVSTSKPPEAS STQEVGKHDSFSTEKQASQEEEDMFYAFVIL HAEEDSEEAVRLKSRLESISSTIGATFSEDFA VPGQSTFRSVEDAIENSAYVMLLLTPNFNTH LNETNADSALMNSIEKPHKHNTVIPLLPRAN GLTRNQMPFILRTKNPLVETRDRDTFEKMA KKVLDLRNIQRQKSMWTEAQLVKKQREKQ QWLQEKKRYCKDFIQESPRVRELEEQIQQLK MQQQHLQPPYAQQTNSHQGFPGRPQSSGPM PFRSPSPMPSYYSGNMWPQLPSNIHIQNAKCI MIGNNSTMTVGGGVDSGDEDNF (GenBank: NP_001187154.1) (Baoprasertkul et al., 2006)
1	0	5	-5	104	Yes	Yes	MAEETELVDEKKPTELCVNRNTVVNSPLER SISGSRSTENISGEHTAAECLIMGASLQPRRE STEAFKVQKEPPKRDDSYPSSLRSTFVQLLQ PRNQRIHSHNQ

Table 2. *Continued*.

Mutation	Insertion	Deletion	Net change (Δ)	Protein length (amino acid)	Frameshift changed	Premature stop codon	Predicted protein sequence
2	5	40	-35	94	Yes	Yes	MAEETELVDEKKPTELCVNRNTVVNSPLERSISGSRSTENISGEHTAAECLIMGASLQPRRESTEAFKVQKEPPKRDDSYPSSLRSTSNKSHNQ
3	0	87	-87	491	No	Yes	MAEETELVDEKKPTELCVNRNTVVNSPLERSISGSRSTENISGEHTAAECLIMGASLQPRRESTEAFKVQKEPPKRDDSYPSSLRSTSKTPPESRDGQNHEAKPSSPLVDHKPFYSVTKSYKSSDPTPLQERAQLEKFKFRQYPDKHDTSEIVTPKSPNIESGLEKTFLSIPSGGGKVGAQPVSTSKPPEASSTQEVGKHDSFSTEKQASQEEEDMFYAFVILHAEEDSEEAVRLKSRLESISSTIGATFSEDFAVPGQSTFRSVEDAIENSAYVMLLLTPNFNTHLNETNADSALMNSIEKPHKHNTVIPLLPRANGLTRNQMPFILRTKNPLVETRDRDTFEKMAKKVLDLRNIQRQKSMWTEAQLVKKQREKQQWLQEKKRYCKDFIQESPRVRELEEQIQQLKMQQQHLQPPYAQQTNSHQGFPGRPQSSGPMPFRSPSPMPSYYSGNMWPQLPSNIHIQNAKCIMIGNNSTMTVGGGVDSGDEDNF

Table 2. *Continued.*

Mutation	Insertion	Deletion	Net change (Δ)	Protein length (amino acid)	Frameshift changed	Premature stop codon	Predicted protein sequence
4	3	131	-128	95	Yes	Yes	MAEETELVDEKKPTELCVNRNTVV NSPLERSISGSRSTENISGEHTAAECL IMGASLQPRRESTEAFKVQKEPPKR DDSYPSSLRSTSRAFKSPG
5	1	0	+1	106	Yes	Yes	MAEETELVDEKKPTELCVNRNTVV NSPLERSISGSRSTENISGEHTAAECL IMGASLQPRRESTEAFKVQKEPPKR DDSYPSSLRSTSIFVQLLQPRNQRIHS HNQ
6	1	0	+1	106	Yes	Yes	MAEETELVDEKKPTELCVNRNTVV NSPLERSISGSRSTENISGEHTAAECL IMGASLQPRRESTEAFKVQKEPPKR DDSYPSSLRSTSTFVQLLQPRNQRIH SHNQ
7	14	0	+14	100	Yes	Yes	MAEETELVDEKKPTELCVNRNTVV NSPLERSISGSRSTENISGEHTAAECL IMGASLQPRRESTEAFKVQKEPPKR DDSYPSSLRSTSRSTPPPRAAPTA
8	35	9	+26	91	Yes	Yes	MAEETELVDEKKPTELCVNRNTVV NSPLERSISGSRSTENISGEHTAAECL IMGASLQPRRESTEAFKVQKEPPKR DDSYPSSLRSTSTQV
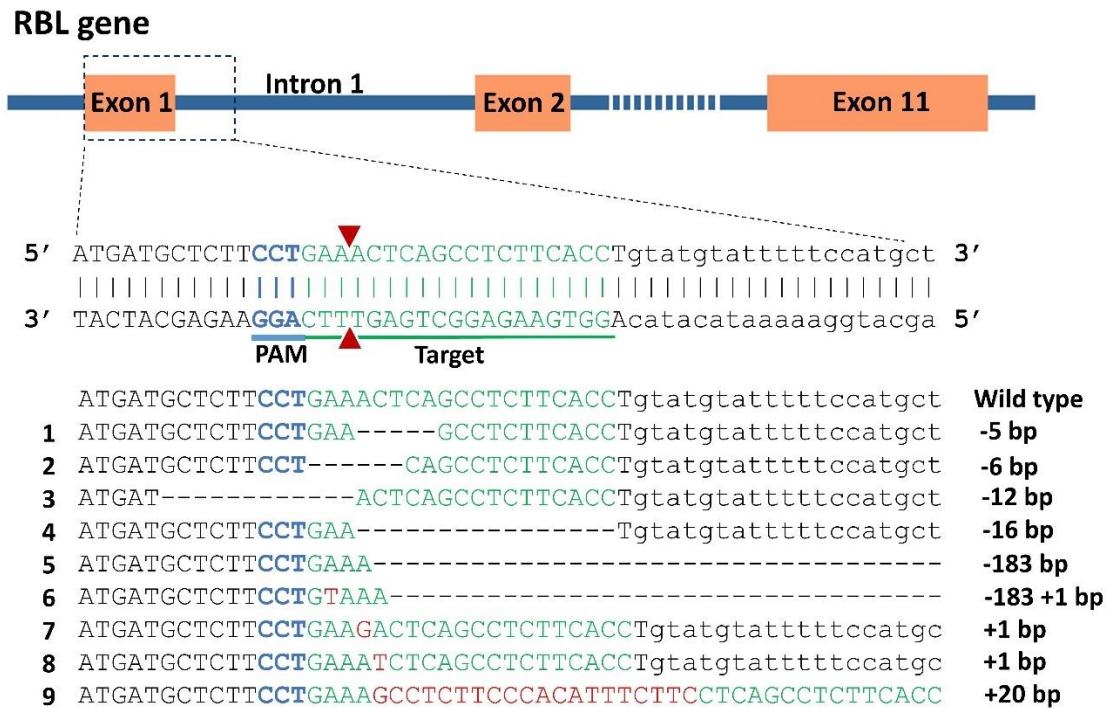
Figure 5. CRISPR/Cas9 induced mutations in rhamnose binding lectin (RBL) gene of channel catfish, *Ictalurus punctatus*. Exonic sequences are UPPERCASE while intronic sequences are lowercase letters. Blue sequence represents the protospacer adjacent motif (PAM) while the green sequence represents the target for gRNA. Double strand break induced by Cas9 protein was expected to occur at the site of the 2 red triangles. Mutations are assigned numbers 1 through 9. Deletion mutations are represented by a dashed line in which each dash corresponds to a nucleotide that has been deleted. Red sequences are insertions.

Table 3. Effects of indel mutations in the rhamnose binding lectin (RBL) gene of channel catfish, *Ictalurus punctatus*, on predicted protein length (amino acids) and sequence compared to the wild type sequence (WT).

Mutation	Insertion	Deletion	Net change (Δ)	Protein length (amino acid)	Frameshift changed	Premature stop codon	Predicted protein sequence
WT	0	0	0	308	No	No	MMLFLKLSLFTLIISAPGLMVSGENM ITCYSDVQRLTCETGLIKVKSTVYGR TNSITCNTNRPFSEVTFTNCALRITTI ADRCNGLKECELKTDLLGNPDPCFG TYKYYNTTYDCINGHRVVICEQGYS TLDCGSDSIEIINANYGRGNSRTCSN GILSSQTQNTNCYAPNTLSIVAAMCK GKKTCTVEASNTIFNDPCVGTVKYL TVSYICTREIVTCESSTATLNCGAHRI KIISANYGRTDSTTCSSGRPASQTSN KNCYTPDALNKIAARCEEQSSCEVP ATNVVFSDPCFGTYKYLTIVYSCV (GenBank: AHJ14694.1) (Thongda et al., 2014)
1.	0	5	-5	29	Yes	Yes	MMLFLKPLHLNYFSSWLNGFWREY DYLLQ

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Table 3. *Continued.*

Mutation	Insertion	Deletion	Net change (Δ)	Protein length (amino acid)	Frameshift changed	Premature stop codon	Predicted protein sequence
2.	0	6	-6	306	No	No	MMLFLSLFTLIISAPGLMVSGENMIT CYSDVQRLTCETGLIKVKSTVYGRT NSITCNTNRPFSEVTFTNCALRITTIA DRCNGLKECELKTDLLGNPDPCFGT YKYYNTTYDCINGHRVVICEQGYST LDCGSDSIEIINANYGRGNSRTCSNGI LSSQTQNTNCYAPNTLSIVAAMCKG KKTCTVEASNTIFNDPCVGTVKYLT VSYICTREIVTCESSTATLNCGAHRIK IISANYGRTDSTTCSSGRPASQTSNK NCYTPDALNKIAARCEEQSSCEVPAT NVVFSDPCFGTYKYLTIVYSCV
3.	0	12	-12	304	No	No	MILSLFTLIISAPGLMVSGENMITCYS DVQRLTCETGLIKVKSTVYGRTNSIT CNTNRPFSEVTFTNCALRITTIADRC NGLKECELKTDLLGNPDPCFGTYKY YNTTYDCINGHRVVICEQGYSTLDC GSDSIEIINANYGRGNSRTCSNGILSS QTQNTNCYAPNTLSIVAAMCKGKKT CTVEASNTIFNDPCVGTVKYLTVSYI CTREIVTCESSTATLNCGAHRIKIISA NYGRTDSTTCSSGRPASQTSNKNCY TPDALNKIAARCEEQSSCEVPATNVV FSDPCFGTYKYLTIVYSCV

Table 3. *Continued.*

Mutation	Insertion	Deletion	Net change (Δ)	Protein length (amino acid)	Frameshift changed	Premature stop codon	Predicted protein sequence
4.	0	16	-16	6	Yes	Yes	MMLFLN
5.	0	183	-183	8	No	Yes	MMLFLKRI (Assuming splicing is not altered)
6.	1	183	-182	5	Yes	Yes	MMLFL (Assuming splicing is not altered)
7.	1	0	+1	31	Yes	Yes	MMLFLKTQPLHLNYFSSWLNGFWR EYDYLLQ
8.	1	0	+1	31	Yes	Yes	MMLFLKSQPLHLNYFSSWLNGFWR EYDYLLQ
9.	20	0	+20	18	Yes	Yes	MMLFLKASSHISSSASSP

4. Discussion

A detailed protocol for microinjection of channel catfish embryos to achieve gene knockout was presented. Injection of CRISPR/Cas9 protein in the yolk is much simpler, time saving, does not require extensive training and proved to be successful for inducing indels in channel catfish TICAM 1 and RBL genes. These procedures lay the foundations for generating gene knockouts in channel catfish using a more reliable and effective method. The protocol can be modified to target other genes in the channel catfish genome using CRISPR/Cas9 system. It is also worth evaluation in other catfish species such as the blue catfish (*Ictalurus furcatus*).

However, if other biologically active substances such as DNA or mRNA are to be injected, the protocol may need to be optimized to determine the best conditions.

In protocols for microinjection of fish embryos, microinjection procedures could be divided into two main categories: injection into the embryonic cells and injection into the yolk. In medaka, morpholinos were injected in the cytoplasm of one-cell embryos to study various developmental processes *in vivo* (Porazinski et al., 2010). In channel catfish, CRISPR/Cas9 mRNA targeting the GnRH gene were microinjected into the blastodisc of one cell embryos (Qin, 2015). In three-spined stickleback (*Gasterosteus aculeatus*), blastomeres were microinjected to generate transgenics and gene knockouts (Erickson et al., 2016). On the other hand, one-cell zebrafish embryos were yolk-injected with morpholinos to manipulate the protein Heart of Glass (Heg) which proved to be effective (Rosen et al., 2009). Approaches of the 2 main categories have advantages and disadvantages, but if both prove to have the same effectiveness for the same species, yolk microinjection of one-cell embryos is preferred for several reasons.

Yolk microinjection is less invasive to the embryos since the microinjection needles do not pierce the embryonic cells. In addition, many embryos can be injected in a short period while the embryos are still at the first cell stage. There is no need to align the embryos at a certain position as is necessary when embryonic cells are injected. This also would save time and reduce the handling stress of embryos. In seasonally spawning fish, there is a limited spawning time in which all the procedures need to be done to achieve successful knockout. In channel catfish, the spawning season lasts for about 2 months (Silverstein and Small, 2004) so developing a rapid but effective microinjection protocol will allow microinjection of many embryos to target many genes in this short time period. Fortunately, channel catfish can be artificially spawned (Su et al.,

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2013) to supply enough embryos for microinjection to overcome the high mortality due to microinjection procedures. Effects of microinjection of gRNA/Cas9 protein on embryo survival are discussed in detail in chapter 3. In addition, the time of fertilization can be controlled to ensure the embryos are in the one-cell stage when needed.

Microinjection of CRISPR/Cas9 induced indels in different fish species including zebrafish (Jao et al., 2013, Chang et al., 2013), Atlantic salmon (*Salmo salar* L.) (Edvardsen et al., 2014), Nile tilapia (*Oreochromis niloticus*) (Li et al., 2014), channel catfish (Qin, 2015), Atlantic killifish (*Fundulus heteroclitus*) (Aluru et al., 2015) and common carp (Zhong et al., 2016). In this protocol, successful indels were induced in the coding sequences with dramatic effects on the predicted protein sequence in most cases, including frameshift mutations or a truncation of the protein due to a premature stop codon. The phenotypic effects of these mutations still need to be investigated. Truncated mRNAs may be degraded through the nonsense-mediated mRNA decay (NMD) pathway (Gatfield et al., 2003) resulting in reduction or inhibition of expression, and if expressed, truncated proteins will likely be nonfunctional (Lim et al., 1997).

In RBL gene, two types of mutations (number 5 and 6) resulted in deletion of 183 bp completely removing half of exon 1, intron 1, exon 2 and part of intron 2. Theoretically, this type of mutation may alter the splicing of RBL pre-mRNA. Mutations at the splice sites inhibit the ability of the spliceosome to recognize the exon (Talerico and Berget, 1990).

5. Conclusion

Development of a reliable efficient protocol for targeted gene editing in channel catfish is crucial for studying functional genomics especially after the genomic resources have been enriched with the sequencing of channel catfish genome. The procedures described here are simple, rapid but efficient and proved to successfully achieve gene knockout. Two genes, TICAM 1 and RBL were targeted to demonstrate the efficiency of CRISPR/Cas9 knockout using microinjection. Depending on what is being injected, the protocol can be modified to include other biologically active substances. It can be also modified to microinject other catfish species or other fish species that have similar egg structure and composition. This protocol lays the foundations for gene knockout in channel catfish.

6. References

Aluru, N., Karchner, S.I., Franks, D.G., Nacci, D., Champlin, D. & Hahn, M.E. (2015). Targeted mutagenesis of aryl hydrocarbon receptor 2a and 2b genes in Atlantic killifish (*Fundulus heteroclitus*). *Aquatic Toxicology,* 158**:** 192-201.

Armstrong, J., Duhon, S. & Malacinski, G. (1989). Raising the axolotl in captivity. *Developmental biology of the axolotl***:** 220-227.

Baoprasertkul, P., Peatman, E., Somridhivej, B. & Liu, Z. (2006). Toll-like receptor 3 and TICAM genes in catfish: species-specific expression profiles following infection with *Edwardsiella ictaluri*. *Immunogenetics,* 58**:** 817-830.

Buono, R. & Linser, P. (1991). Transient expression of RSVCAT in transgenic zebrafish made by electroporation. *Molecular marine biology and biotechnology,* 1**:** 271-275.

Chang, N., Sun, C., Gao, L., Zhu, D., Xu, X., Zhu, X., Xiong, J.-W. & Xi, J.J. (2013). Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. *Cell research,* 23**:** 465-472.

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

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W. & Marraffini, L.A. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science,* 339**:** 819-823.

Cradick, T.J., Fine, E.J., Antico, C.J. & Bao, G. (2013). CRISPR/Cas9 systems targeting β-globin and CCR5 genes have substantial off-target activity. *Nucleic acids research***:** gkt714.

Davis, B., Brown, D., Prokopishyn, N. & Yannariello-Brown, J. (2000). Micro-injection-mediated hematopoietic stem cell gene therapy. *Current opinion in molecular therapeutics,* 2**:** 412-419.

Doyon, Y., Mccammon, J.M., Miller, J.C., Faraji, F., Ngo, C., Katibah, G.E., Amora, R., Hocking, T.D., Zhang, L. & Rebar, E.J. (2008). Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nature biotechnology,* 26**:** 702-708.

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

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

Dunham, R.A., Warr, G.W., Nichols, A., Duncan, P.L., Argue, B., Middleton, D. & Kucuktas, H. (2002). Enhanced bacterial disease resistance of transgenic channel catfish *Ictalurus punctatus* possessing cecropin genes. *Mar Biotechnol (NY),* 4**:** 338-44. DOI 10.1007/s10126-002-0024-y

Dunham, R.A. & Winn, R.N. (2014). Production of transgenic fish. *Transgenic animal technology: a laboratory handbook.* Elsevier BV, Amsterdam.

Edvardsen, R.B., Leininger, S., Kleppe, L., Skaftnesmo, K.O. & Wargelius, A. (2014). Targeted mutagenesis in Atlantic salmon (*Salmo salar* L.) using the CRISPR/Cas9 system induces complete knockout individuals in the F0 generation.

Erickson, P.A., Ellis, N.A. & Miller, C.T. (2016). Microinjection for Transgenesis and Genome Editing in Threespine Sticklebacks. *JoVE (Journal of Visualized Experiments)***:** e54055-e54055.

Gatfield, D., Unterholzner, L., Ciccarelli, F.D., Bork, P. & Izaurralde, E. (2003). Nonsense-mediated mRNA decay in Drosophila: at the intersection of the yeast and mammalian pathways. *The EMBO journal,* 22**:** 3960-3970.

Hammer, R.E., Pursel, V.G., Rexroad, C.E., Wall, R.J., Bolt, D.J., Ebert, K.M., Palmiter, R.D. & Brinster, R.L. (1985). Production of transgenic rabbits, sheep and pigs by microinjection. *Nature,* 315**:** 680 - 683.

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

Inoue, K., Yamashita, S., Hata, J.-I., Kabeno, S., Asada, S., Nagahisa, E. & Fujita, T. (1990). Electroporation as a new technique for producing transgenic fish. *Cell differentiation and development,* 29**:** 123-128.

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

Komarova, Y., Peloquin, J. & Borisy, G. (2011). Components of a microinjection system. *Cold Spring Harbor Protocols,* 2011**:** pdb. ip27.

Li, M., Yang, H., Zhao, J., Fang, L., Shi, H., Li, M., Sun, Y., Zhang, X., Jiang, D. & Zhou, L. (2014). Efficient and heritable gene targeting in tilapia by CRISPR/Cas9. *Genetics,* 197**:** 591-599.

Liang, X., Potter, J., Kumar, S., Zou, Y., Quintanilla, R., Sridharan, M., Carte, J., Chen, W., Roark, N. & Ranganathan, S. (2015). Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *Journal of biotechnology,* 208**:** 44-53.

Lim, J., Ghadessy, F.J. & Yong, E. (1997). A novel splice site mutation in the androgen receptor gene results in exon skipping and a non-functional truncated protein. *Molecular and cellular endocrinology,* 131**:** 205-210.

Liu, Z., Liu, S., Yao, J., Bao, L., Zhang, J., Li, Y., Jiang, C., Sun, L., Wang, R. & Zhang, Y. (2016). The channel catfish genome sequence provides insights into the evolution of scale formation in teleosts. *Nature communications,* 7.

Lu, J.-K., Fu, B.-H., Wu, J.-L. & Chen, T.T. (2002). Production of transgenic silver sea bream (*Sparus sarba*) by different gene transfer methods. *Marine biotechnology,* 4**:** 328-337.

Miller, J.C., Tan, S., Qiao, G., Barlow, K.A., Wang, J., Xia, D.F., Meng, X., Paschon, D.E., Leung, E. & Hinkley, S.J. (2011). A TALE nuclease architecture for efficient genome editing. *Nature biotechnology,* 29**:** 143-148.

Neumann, E., Schaefer-Ridder, M., Wang, Y. & Hofschneider, P. (1982). Gene transfer into mouse lyoma cells by electroporation in high electric fields. *The EMBO journal,* 1**:** 841.

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

Porazinski, S.R., Wang, H. & Furutani-Seiki, M. (2010). Microinjection of medaka embryos for use as a model genetic organism. *Journal of visualized experiments: JoVE.*

Powers, D.A., Hereford, L., Cole, T., Chen, T.T., Lin, C., Kight, K., Creech, K. & Dunham, R. (1991). Electroporation: a method for transferring genes into the gametes of zebrafish (*Brachydanio rerio*), channel catfish (Ictalurus punctatus), and common carp (*Cyprinus carpio*). *Molecular marine biology and biotechnology,* 1**:** 301-308.

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

Qin, Z., Li, Y., Su, B., Cheng, Q., Ye, Z., Perera, D.A., Fobes, M., Shang, M. & Dunham, R.A. (2016). Editing of the Luteinizing Hormone Gene to Sterilize Channel Catfish, *Ictalurus punctatus*, Using a Modified Zinc Finger Nuclease Technology with Electroporation. *Marine Biotechnology,* 18**:** 255-263.

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

Rosen, J.N., Sweeney, M.F. & Mably, J.D. (2009). Microinjection of zebrafish embryos to analyze gene function. *JoVE (Journal of Visualized Experiments)***:** e1115-e1115.

Silverstein, J.T. & Small, B.C. (2004). Reproductive physiology. IN Tucker, C.S. & Hargreaves, J.A. (Eds.) *Biology and culture of channel catfish.* Elsevier.

Sin, F., Bartley, A., Walker, S., Sin, I., Symonds, J., Hawke, L. & Hopkins, C. (1993). Gene transfer in chinook salmon (*Oncorhynchus tshawytscha*) by electroporating sperm in the presence of pRSV-lacZ DNA. *Aquaculture,* 117**:** 57-69.

Su, B., Perera, D.A., Zohar, Y., Abraham, E., Stubblefield, J., Fobes, M., Beam, R., Argue, B., Ligeon, C. & Padi, J. (2013). Relative effectiveness of carp pituitary extract, luteininzing hormone releasing hormone analog (LHRHa) injections and LHRHa implants for producing hybrid catfish fry. *Aquaculture,* 372**:** 133-136.

Talerico, M. & Berget, S.M. (1990). Effect of 5'splice site mutations on splicing of the preceding intron. *Molecular and cellular biology,* 10**:** 6299-6305.

Thongda, W., Li, C., Luo, Y., Beck, B.H. & Peatman, E. (2014). L-rhamnose-binding lectins (RBLs) in channel catfish, *Ictalurus punctatus*: Characterization and expression profiling in mucosal tissues. *Developmental & Comparative Immunology,* 44**:** 320-331.

Treen, N., Yoshida, K., Sakuma, T., Sasaki, H., Kawai, N., Yamamoto, T. & Sasakura, Y. (2014). Tissue-specific and ubiquitous gene knockouts by TALEN electroporation provide new approaches to investigating gene function in Ciona. *Development,* 141**:** 481-487.

Varshney, G.K., Pei, W., Lafave, M.C., Idol, J., Xu, L., Gallardo, V., Carrington, B., Bishop, K., Jones, M. & Li, M. (2015). High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. *Genome research,* 25**:** 1030-1042.

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

Chapter 3: Effects of microinjection of different dosages of CRISPR/Cas9 protein on mutation rate, embryonic development, hatchability and early fry survival in channel catfish, *Ictalurus punctatus*.

Abstract

The current study was conducted to assess the effects of microinjection of different dosages of guide RNA (gRNA)/Cas9 protein on the mutation rate, embryo mortality, hatchability and early fry survival in channel catfish, *Ictalurus punctatus*. Guide RNA targeting two of the channel catfish immune related genes, toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene, were microinjected into the yolk of one-cell embryos. Three dosages of gRNA/Cas9 protein (low, 2.5 ng gRNA/7.5 ng Cas9 protein, medium, 5 ng gRNA/15 ng Cas9 protein and high, 7.5 ng gRNA/22.5 ng Cas9 protein) were injected into the yolk of one-cell embryos. Two types of control embryos were used; injected control (injected with the same solution without gRNA or Cas9 protein, iCTRL) and non-injected control (nCTRL) embryos. Embryos were incubated in Holtfreter's solution with continuous aeration. Dead embryos were recorded and removed daily. Time of hatch and number of dead fry were recorded. Mutated individuals were identified with the Surveyor® mutation detection assay and confirmed with DNA sequencing. In both genes, microinjection procedure itself increased the embryo mortality when the iCTRL group was compared to the nCTRL group ($p < 0.05$). Mutation rate increased by increasing the gRNA/Cas9 dosage ($p < 0.05$). Mutation rate was higher in dead embryos compared to 4-month-old fingerlings but it was not significant ($p > 0.05$).

In TICAM 1 microinjected embryos, the low dosage did not significantly increase the embryo mortality when compared to the iCTRL ($p = 0.295$). The medium and high dosages increased the embryo mortality significantly when compared to the iCTRL ($p < 0.0001$). However, the embryo mortality was not statistically different among the medium and high dosages ($p = 0.892$). All TICAM 1 treatments had similar mean time to hatch ($p = 0.130$). Hatching percent was significantly lower in the medium ($p = 0.009$) and high dosages ($p = 0.005$) when compared to the nCTRL group. All other comparisons were not significant ($p > 0.05$). Survival % was significantly lower in the iCTRL group when compared to the nCTRL group ($p = 0.001$) while all other groups had statistically similar hatch % ($p > 0.05$). The fry in the nCTRL group had the longest mean time to death compared to the other 4 groups ($p < 0.05$) in which no differences in the fry mean survival time to death were detected ($p > 0.05$).

In RBL, the lowest embryo mortality percent (13.1%) was recorded in the nCTRL group ($p < 0.05$) followed by the iCTRL group (29.8% mortality, $p < 0.05$). Treatment groups had significantly higher embryo mortality rates when compared to nCTRL and iCTRL ($p = 0.0001$). Medium dosage resulted in higher mortality than the low dosage ($p = 0.044$). No significant differences in embryo mortality percent were detected between the medium and high dosage groups ($p = 0.999$). The nCTRL group had the longest embryo mean time to death followed by the iCTRL group, both of which were statistically different ($p < 0.0001$). Both nCTRL and iCTRL groups had significantly longer embryo mean time to death compared to the three treatment groups ($p < 0.0001$), which had similar means ($p > 0.05$). The highest hatching % was recorded in the nCTRL group then iCTRL followed by low dosage and finally the medium and high dosages. Hatching % was statistically different between all the groups ($p < 0.05$) except the medium and high dosages which were similar ($p = 1.000$). Effects on mean time to hatch were:

nCTRL and iCTRL hatched earlier than the three treatment groups ($p < 0.0001$), low and medium dosages hatched at the same time ($p = 0.184$). Also medium and high dosages had statistically similar hatching time ($p = 0.628$). Low dosage embryos hatched earlier than high dosage embryos ($p = 0.039$). The nCTRL and iCTRL had similar mean fry survival time ($p = 0.170$) and the 2 control groups had higher mean survival time for the fry than treatment groups ($p < 0.05$). All treatment groups had similar mean survival time for the fry ($p > 0.05$). The current results lay the foundations for designing gene editing experiments in channel catfish and can be used as a guide for other fish species.

1. Introduction

Channel catfish (*Ictalurus punctatus*) is an important fish species in the United States that has been extensively used in aquaculture, scientific research and is extremely popular among anglers (Jackson, 2004). Channel catfish has been hybridized with blue catfish (*Ictalurus furcatus*) to produce the channel catfish female X blue catfish male (CB) hybrids with superior performance when compared to both parent species (Dunham and Masser, 2012). Catfish production has been contracting in the US since its peak in 2003 when more than 300,000 tonnes were processed (Hanson and Sites, 2015). Restoration of catfish production in the US would require integration of both management and genetic improvement programs including traditional breeding and molecular genetics tools (Dunham, 2011). The benefits from combining these enhancement programs would be maximized with the use of catfish genomic resources (Liu et al., 2016a).

Recently, extensive genomic data for channel catfish became available after the complete sequencing of its genome (Liu et al., 2016b). However, to implement this genomic data in genetic enhancement programs, it would be necessary to perform functional studies for economically important traits such as growth, feed conversion, tolerance to hypoxia, disease resistance and reproduction. One way to study functional genomics is gene editing using the CRISPR/Cas9 system (Zhou et al., 2014). The development of CRISPR/Cas9 system made the genome editing experiments more efficient, precise, rapid and economic. The system consists of a guide RNA which determines the targeted sequence in the genome and a DNA endonuclease enzyme, Cas9 (Cong et al., 2013). Since 2013, many gene editing experiments have been conducted on fish species including gene knockout (Jao et al., 2013, Edvardsen et al., 2014, Ablain et al., 2015, Varshney et al., 2015) and gene insertion or knock-in (Auer et al., 2014, Irion et al., 2014, Kimura et al., 2014, Hisano et al., 2015).

Knocking out or silencing a gene may affect not only the primary phenotype, but also one or more secondary phenotypes due to the interactions of gene products in different metabolic pathways resulting in pleiotropic effects. (Ho et al., 1997, Söker et al., 2008, Mohandas et al., 2016). In medaka (*Oryzias latipes*), successful knockout of myostatin (MSTN) gene increased the expression level of myogenic regulatory factors (MyoD, Myf5 and myogenin) (Chisada et al., 2011, Chiang et al., 2015). As a trade-off for faster growth demonstrated by increased body length and weight in MSTN-deficient medaka, the immune system was compromised. Following immersion challenge of F_3 MSTN-mutant medaka with red spotted grouper nervous necrosis virus (RGNNV), although both mutant and wild type fish had the same mortality percent, MSTN-deficient fish had lower expression of several interferon-stimulated genes and higher virus copy numbers (Chiang et al., 2015).

Microinjection of guide RNA (gRNA)/Cas9 protein into catfish embryos may affect the hatching, embryo development and early fry survival. The higher mortality in injected embryos may be due to the microinjection procedure itself, off target mutations induced by the gRNA/Cas9 protein (Cradick et al., 2013) or pleiotropic effects of target gene knockout. The extent of off-target mutations depends on the specificity of the CRISPR/Cas9 which is determined mainly by the protospacer adjacent motif (PAM) and the guide RNA sequence (Mali et al., 2013, Wu et al., 2014). Off-target sites with 5 nucleotides mismatch with the gRNA sequence can still be targeted at a frequency that is comparable to on-target site (Fu et al., 2013). However, off target mutations may be minimized by better gRNA design (Pattanayak et al., 2013, Cho et al., 2014, Lee et al., 2016), optimization of the dosage of gRNA/Cas9 protein (Hsu et al., 2013) and the use of Cas9 nickase mutant with paired gRNAs which reduced off-target effects by 50 to 1,500 fold in cell lines (Ran et al., 2013, Cho et al., 2014)

Since the CRISPR/Cas9 technology opened avenues for precise genome editing, it is important to assess the effects of gRNA and Cas9 on the survival and hatchability of microinjected catfish embryos to allow better experimental design for gene editing experiments. Studying such effects would allow accurate estimation of the number of embryos and the amount of gRNA/Cas9 protein to be injected to generate enough founder fish for functional genomics studies with minimal off-target mutations. So, the objective of the current study was to assess the effects of microinjection of different dosages of gRNA/Cas9 protein on the embryo survival, embryonic development, hatching percent and early fry survival in channel catfish. Guide RNAs used in this study were designed to target the channel catfish toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene.

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2. Materials and Methods

This experiment was conducted at the Fish Genetics Research Unit, E. W. Shell Fisheries Research Center, Auburn University, AL. All experimental protocols used in this experiment were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC) before the experiment was initiated.

2.1. Experimental design

The experiment included three treatment and two control groups for TICAM 1 and RBL genes. Three dosages of gRNA/Cas9 protein (2.5 ng gRNA/7.5 ng Cas9 protein, 5 ng gRNA/15 ng Cas9 protein and 7.5 ng gRNA/22.5 ng Cas9 protein) were injected (with fixed volume, 50 nL), while the 4th group was injected with the same volume (50 nL) without gRNA or Cas9 protein as shown in Table 4. The second control was not microinjected. Each of the treatment and control groups for the 2 genes had three replicates. Brood stock preparation, artificial spawning and microinjection procedures were performed as described in details in chapter 2.

2.2. Guide RNA and Cas9 protein

Guide RNAs used in the study are listed in

Table 5. Four gRNAs for TICAM 1 and 5 gRNAs for RBL were designed using CRISPRscan gRNA design tool (Moreno-Mateos et al., 2015). Guide RNAs were prepared per Shah et al. (2015) with the use of Expand High Fidelity[PLUS] PCR System (Roche Diagnostics, USA). Cas9 protein was purchased from PNABio, CA, USA. For TICAM 1 gene, equal amounts of the 4 gRNAs were mixed with Cas9 protein before injection. For RBL, equal amounts of the 5 gRNAs were mixed with Cas9 protein before injection. For each dosage, the concentrations of gRNAs and Cas9 proteins were adjusted so that the injection of 50 nl will deliver approximately the amount needed for each dosage.

Table 4 Different treatments and control groups of channel catfish, *Ictalurus punctatus*, embryos microinjected with gRNA/Cas9 protein targeting toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene. The solution injected into the iCTRL embryos had the same composition (water with up to one third of 0.5% phenol red solution) as the treatment groups but without gRNA/Cas9 protein.

Treatment	Guide RNA (ng)		Cas9 protein (ng)		Total volume injected (nL)	
	TICAM 1	RBL	TICAM 1	RBL	TICAM 1	RBL
Low dosage	2.5	2.5	7.5	7.5	50	50
Medium dosage	5	5	15	15	50	50
High dosage	7.5	7.5	22.5	22.5	50	50
Injected control (iCTRL)	0	0	0	0	50	50
Non-injected control (nCTRL)	0	0	0	0	0	0

Table 5 The genomic target sequences for gRNAs used to target the channel catfish, *Ictalurus punctatus* toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) coding sequence and rhamnose binding lectin (RBL) genes. Underlined sequences represent the protospacer adjacent motif (PAM).

Target sequence for gRNA (3'→5')	
TICAM 1	RBL
1. GGAGGTGAAGCACGTCGAGGA	1. GGACTTTGAGTCGGAGAAGTGG
2. GGTGACGGAGATTTCTGTGGCGG	2. GGGGCTGGGTACGAAACCTTGG
3. GGGTTCTCGGGTTTATATCTT	3. GGTGAATGCTGACGTAGTTAC
4. GGTGGACTTCGTAGGAGATGAG	4. GGAGTCAAGAGTCTGGGTCTTG
	5. GGAGTGACACAGGATGTAGACG

2.3. Embryo incubation and hatching

Immediately after microinjection, embryos were incubated in Holtfreter's solution (59 mM NaCl, 0.67 mM KCl, 2.4 mM NaHCO$_3$, 0.76 mM CaCl$_2$, 1.67 mM MgSO$_4$) (Armstrong et al., 1989) with 100 ppm doxycycline (Su et al., 2015). Each replicate of a treatment was incubated in a plastic tub containing 7 liters of Holtfreter's solution and continuous aeration as shown in Figure 6.
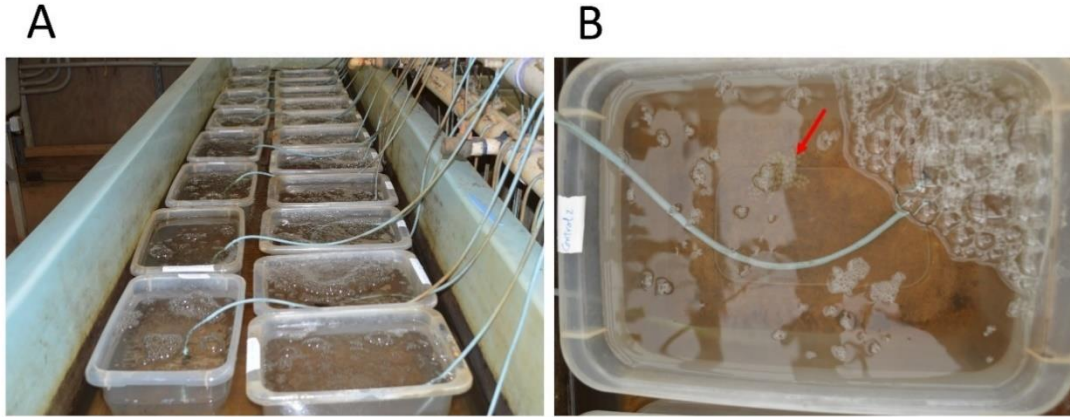
Figure 6 The static incubation of channel catfish, *Ictalurus punctatus*, embryos microinjected with gRNA/Cas9 protein targeting toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) coding sequence and rhamnose binding lectin (RBL) genes. A: Embryos were incubated in replicate plastic tubs containing Holtfreter's solution with 10 ppm doxycycline and continuous aeration. Tubs were placed in a rectangular tank containing water. B: Red arrow points to the non-injected control (nCTRL) channel catfish embryos during the incubation.

Incubation temperature ranged from 26 to 28°C while dissolved oxygen levels were kept above 5 ppm using air supplied from air blowers. All tubs for each gene were held in a rectangular tank containing pond water (Figure 6) to reduce the fluctuations in temperatures and ensure all the experimental units had the same temperature. The number of embryos in each replicate for TICAM 1 and RBL can be found in Table 6.

Table 6 Number of channel catfish (*Ictalurus punctatus*) embryos microinjected with different dosages of gRNA/Cas9 protein targeting toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene and their controls. Three replicates were used for each of the treatment and control groups for each gene.

| Treatment | Number of injected embryos in each of the 3 replicates (R1,2,3) | | | | | | | |
| | TICAM 1 | | | | RBL | | | |
	R1	R2	R3	Total	R1	R2	R3	Total
Low	52	50	38	140	67	47	52	166
Medium	50	42	34	126	34	52	38	124
High	38	30	40	108	42	65	66	173
Injected control (iCTRL)	54	54	51	159	42	34	38	114
Non-injected control (nCTRL)	80	86	97	263	71	71	102	244

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. Temperature of Holtfreter's solution was monitored with a thermometer and adjusted to the same degree as the old solution to minimize the adverse effects of temperature fluctuation on embryos. When hatching began, the time of hatch, number of hatching fry and number of dead fry were recorded separately for each experimental replicate. After hatch, the fry were reared in Holtfreter's solution without doxycycline until 10 days post fertilization (dpf). When the fry began to swim up (at 9-10 dpf), they were fed artemia nauplii (Pentair Aquatic Eco-systems, USA) three times/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 Holtfreter's solution was replaced

with pond water. Water quality parameters were monitored daily. The experiment was

terminated at 18 dpf when no fry died in the 5 groups for each gene for 5 successive days.

2.4. Mutation detection

Mutation detection, calculation of mutation rates and identification of different types of

mutations were investigated at the binding sites for all TICAM 1 gRNAs and 2 gRNAs for RBL

(no 4 and 5,

Table 5

Table 5). Genomic DNA was extracted from both whole single embryos that died after 72

h post fertilization and barbel tissue from 3-4 month old fingerlings. Yolk and egg shells were

removed from the embryos before DNA was extracted. DNA extraction was performed using proteinase K digestion and ethanol precipitation as described in chapter 4. PCR and Surveyor® mutation detection assay were performed to identify the mutant embryos and fingerlings. Primers (PCR was performed using Expand High Fidelity[PLUS] PCR System (Roche Diagnostics, Indianapolis, IN, USA) with the following components: up to 20 µl PCR grade water; 1X Expand HiFi[PLUS] reaction buffer with $MgCl_2$, 0.2 mM dNTP mix, 0.4 µM for each of the forward and reverse primer of the same set, 100-300 ng genomic DNA and 1.25 units of Expand HiFi[PLUS] enzyme blend. PCR cycling conditions were as follows: initial denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 sec, annealing at temperatures listed in **Error! Not a valid bookmark self-reference.** for 30 sec, extension at 72 °C for 1 min/kb; and final extension at 72 °C for 10 min. Two PCR reactions for each sample were prepared, one was used to detect large deletion while the other was used for Surveyor® mutation detection assay.

Table 7) were designed to amplify a segment TICAM 1 and RBL genes. The annealing site for primers flanked the binding sites for gRNAs by not less than 100 bp from both ends.

PCR was performed using Expand High Fidelity[PLUS] PCR System (Roche Diagnostics, Indianapolis, IN, USA) with the following components: up to 20 µl PCR grade water; 1X Expand HiFi[PLUS] reaction buffer with $MgCl_2$, 0.2 mM dNTP mix, 0.4 µM for each of the forward and reverse primer of the same set, 100-300 ng genomic DNA and 1.25 units of Expand HiFi[PLUS] enzyme blend. PCR cycling conditions were as follows: initial denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 sec, annealing at temperatures listed in **Error! Not a valid bookmark self-reference.** for 30 sec, extension at 72 °C for 1 min/kb; and final extension

at 72 °C for 10 min. Two PCR reactions for each sample were prepared, one was used to detect large deletion while the other was used for Surveyor® mutation detection assay.

Table 7 Primers used to amplify a partial sequence of channel catfish, *Ictalurus punctatus*, toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene flanking the target sites for guide RNAs. Primers T1F and T1R were used to amplify DNA segments containing small indels while primers T2F and T2R were used for large deletion. Primers RBL 1F and RBL 1R were used to amplify a partial DNA sequence

Primer name	Strand	Sequence 5'-3'	Annealing temperature	Product length (bp)
T1F	Forward	GCTGCTGAATGTCTGATTATG	60 °C	750
T1R	Reverse	GTCCTCCACACTCCTGAAG		
T2F	Forward	ACTGGTGGACGAGAAGAAG	60 °C	1462
T2R	Reverse	CTGGATGTGGATGTTGGATG		
RBL F	Forward	ATCGTGTTGTGATCTGTGAG	57 °C	645
RBL R	Reverse	GCCCTAGCCAATTTGATGTT		

To detect large deletions, PCR products were resolved in 2% agarose gel and compared to wild type controls. Samples with large deletion showed shorter DNA bands(s) (Figure 7, Figure 10). Surveyor® mutation detection assay was performed using Surveyor® mutation detection kit for standard gel electrophoresis (Integrated DNA Technologies) according to the manufacturer instructions. Briefly, PCR products were hybridized. Samples from mutated P_1 individuals formed heteroduplexes after hybridization (heteroduplex is a DNA molecule in which one strand is wild type and the other is mutated). Surveyor® nuclease was used to cleave heteroduplexes at the mismatch points (expected mutation site). A negative control was included

in the reaction by using DNA from full sib channel catfish that were not injected with

gRNA/Cas9 protein. Surveyor®-digested samples were electrophoresed for 50 minutes in 2%

agarose gel using 1X TBE and compared to wild type samples (Figure 7, Figure 11).

DNA sequencing:

Since the PCR product was expected to be a mixture of both wild-type and mutated

alleles, cloning of the PCR product before sequencing was necessary. For TICAM 1, PCR was

performed on mutant individuals that were previously identified with PCR or Surveyor® assay

using the primers T2F (forward) and T2R (reverse). For RBL, primers RBL F (forward) and

RBL R (reverse) were used to amplify a segment from mutant individuals for sequencing. PCR

products were then cloned in pCR™4-TOPO® vector using TOPO® TA cloning® kit for

sequencing following the manufacturer instructions (Invitrogen by Life Technologies). DNA

from 3 wild type individuals that were full sib to the mutated individuals was amplified using the

same primers, then pooled into one reaction and cloned as a wild type control for sequencing.

One Shot® TOP10 Electrocomp™ *E. coli* (Invitrogen by Life Technologies) were

transformed with the pCR™4-TOPO® vector containing the PCR product following the

manufacturer's instructions with some modifications. 2 µl of the TOPO® cloning reaction were

added to 25 µl of competent cells, kept on ice for 30 minutes, heat shocked at 42 ºC for 30 sec,

then kept on ice for 2 minutes before adding 250 µl of SOC medium (provided with the kit) and

incubated at 37 ºC for 1 h. Selection of transformed cells was achieved by plating 50 µl and 100

µl on LB agar plates containing 100 ppm ampicillin and overnight incubation at 37°C. A single

colony was picked up and inoculated into a 1.5 ml eppendorf tube containing 1 ml LB medium

with the same concentration of ampicillin and incubation conditions before sending the samples

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for sequencing. T2F primer was used for sequencing of TICAM 1 mutated samples while RBL F primer was used for RBL mutated samples.

Mutation rates were calculated for replicates of different treatments for TICAM 1 and RBL in both dead embryos and alive fingerlings. Mutation rates were calculated as the number of mutated individuals detected by PCR or Surveyor® assay in a replicate or treatment / total number of individuals in the same replicate or treatment * 100.

2.5. Statistical analysis

Since the density of embryos and fry was different between the different groups and even the replicates of the same group, an analysis of covariance (ANCOVA) for the effects of embryo and fry density on embryo mortality, survival, hatching and fry survival was performed. No significant effects for differences in density were detected ($p = 0.274$).

Embryo mortality % was calculated as the number of dead embryos in a group or treatment divided by the total number of embryos and multiplied by 100. Each embryo was assigned a value representing the time (dpf) of death. Survival % was similarly calculated as the number of alive embryos in a group or treatment divided by the total number of embryos and multiplied by 100. Survival curves for embryos in the 5 groups were compared using Kaplan-Meier test. Log Rank (Mantel-Cox) test was used for pairwise comparisons for embryo survival.

Hatching % was calculated as the total number of fry that have completed hatching/total number of embryos*100. Each fry was assigned a day representing the time (dpf) of hatch. Mean time to hatch for each group was compared using Kaplan-Meier test. Hatching percent for the 5 groups was compared using one-way ANOVA. All assumptions were satisfied.

Dead fry were collected daily, recorded and assigned a value representing the day of death (dpf). Survival % was calculated as the number of alive fry/total number of fry (alive + dead) *100. For TICAM 1, when equality of variance for survival percent was not satisfied, Welch's test was used and pairwise comparisons for survival percent among the 5 groups were done using Games-Howell test. To account for the time to death, survival curves of fry in the 5 groups were compared using Kaplan-Meier test and survival curves were plotted. Alive fry were assigned a value (18) representing the dpf in which the experiment was terminated. In the survival analysis, alive fry were censored (the actual survival time is partially known, if the experiment continued, the survival time of alive fish would have changed). Pairwise comparisons for survival time to death were performed using Log Rank (Mantel-Cox) test. Histograms were generated using Microsoft Excel 2016 and all statistical analysis were performed using SPSS 16.0.

3. Results

3.1. Mutation rate

3.1.1. TICAM 1:

PCR detected the large deletion in TICAM 1 gene demonstrated by shorter band(s) when compared to the wild type PCR product (750 bp) as shown in Figure 7A. Mutations in some of the samples that did not show shorter bands with PCR were detected with Surveyor® assay. Mutated samples had multiple bands after surveyor® digestion when compared to the surveyor®-digested wild type control (Figure 7B). There was a positive correlation between the gRNA/Cas9 protein dosage and the mutation rate ($r = 0.445$, $n = 16$, $p = 0.084$) (Table 8, Figure

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8). Mutation rate for the same dosage was higher in dead embryos than 4-month-old fingerlings (Table 8, Figure 8). However, the correlation between mutation rate and status (dead embryos or alive fingerlings) was not significant ($r = 0.337$, n = 16, $p = 0.202$).

DNA sequencing of TICAM 1 pooled samples revealed different types of mutations (Figure 9). Deletion mutations ranged from a few base pairs to more than 900 bp deletion. Insertions ranged from a few bp to 25 bp. In one sequencing reaction, a segment of 70 bp was found inverted (TCCTCTACTCAGGAAGTTGGAAAGCATGACAGCTTTAGTACAGAGA AGCAAGCCAGCCAGGAGGAAGAGG). Three of the 4 gRNA (no 1, 2 and 4) were efficient in inducing mutations. Guide RNA no 3 was not as efficient as other gRNAs (induced mutation in 3 sequencing reaction out of 60, not listed in Figure 9). Each mutation in Figure 9 was found once in 60 sequencing reactions. The rest (36) were wild type.
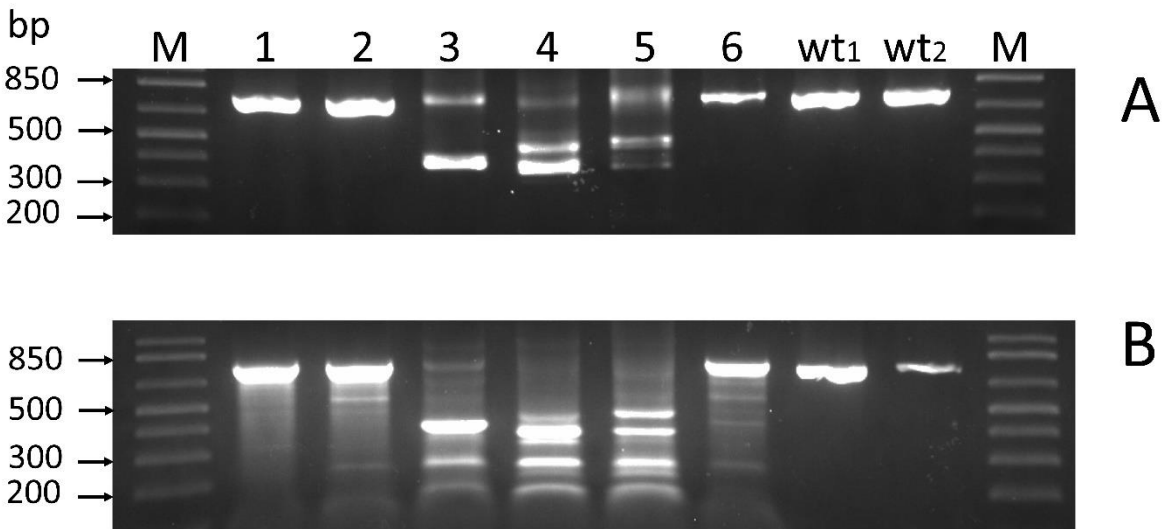
Figure 7 Mutation detection in channel catfish (*Ictalurus punctatus*) embryos microinjected with gRNA/Cas9 protein targeting the channel catfish toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene. For each sample, half of the PCR product was resolved in

2% agarose gel to detect the large deletion (A) while the other half was digested with Surveyor®

enzyme (B). A: PCR amplification of a partial segment of TICAM 1 gene using T1F (forward)

and T1R (reverse) primers. Samples 1-6 came from embryos that were microinjected with

gRNA/Cas9 protein while samples 7 and 8 represent wild type (wt$_1$ and wt$_2$) channel catfish that

were full sib to embryos 1-6. Embryos with large deletion had short band(s) when compared to

the wild type 750 bp band. B: After digestion of the PCR product with Surveyor® enzyme,

samples that had more bands than the wild type controls were considered mutated. Lane M

contained 1 kb plus DNA ladder (Invitrogen).

Figure 8 Mutation rate ± standard error (SE) of channel catfish (*Ictalurus punctatus*) dead

embryos and alive fingerlings microinjected with gRNA/Cas9 protein targeting toll/interleukin 1

receptor domain-containing adapter molecule (TICAM 1) gene. Three dosages were

microinjected into one-cell embryos (low dosage: 2.5 ng gRNA/7.5 ng Cas9, medium dosage: 5

ng gRNA/15 ng Cas9 and high dosage: 7.5 ng gRNA/22.5 ng Cas9).

TICAM 1 coding sequence

ATG   TGA

Indels (bp)

```
5'CCTCCACTTCGTGCAGCTCCTAC//GACCACTGCCTCTAAAGACACCGCCAG//CACCTGAAGCATCCTCTACTCAG 3' WT
5'CCTCCACTTCGTGCAGCTCCTAC//GACCACTGC------------------------------AAGCATCCTCTACTCAG 3'-309
5'CCTCCACTTCGTGCAGCTCCTAC//GACCACTGCCTCT-------CCGCCAG//CACCTGAAGCATCCTCTACTCAG 3'-7
5'CCTCCACTTCGTGCAGCTCCTAC//--------CCTCTAAAGACACCGCCAG//---------------------- 3' -17-64
5'CCTCCACTTCGTGCAGCTCCTAC//------------TAAAGACACCGCCAG//---------------------- 3' -14-367
5'CCTCCACTTCGTGCAGCTCCTAC//GACCACTGCCTC------C-CCGCCAG//CACCTCTAGAAGCA---------- 3' -6-1+3-605
5'CCTCCAGG-------------------------------------//---------------TACTCAG 3' +2-128-39
5'CCTCCACTT---------------ACCA---CCTCTAAAGACACCGCCAG//CACCTGTGTCAACAAGCAAGCAT 3' -73-3+11
5'CCTCCA-----TGCAGCTCCTAC//GACCACTGCCTCTAAAGACACCGCCAG//CACCTGAAGCATCCTCTACTCAG 3' -5
5'CCTCCACTTCGTGCAGCTCCTAC//GACCA------CTAAAGACACCGCCAG//CACCTGCAAGCA----------- 3' -6-712
5'CCTCCACTTCGTGCAGCTCCTAC//GACCACTGCATCTAGACCACTGCTTCCTGAGT---AAGCATCCTCTACTCAG 3' +23-309
5'CCTCCA---------------------------------------------------------------- 3' -741
5'CCTCCACTTCGTGCAGCTCCTAC//GACCACTGC---TAAAGACACCGCCAG//CACCTGAAGCATCCTCTACTCAG 3' -3
5'CCTCCACTTCGTGCAGCTCCTAC//GACCACTGCCTCTAAAGACACCGCCAG//---------CATCCTCTACTCAG 3' -10
5'CCTCCACTTCGTGCAGCTCCTAC//GACCACTG------------------//CAC---------CTCTACTCAG 3' -210 -10
5'CC---------------CTCCTAC//GACCAC----TCTAAAGACACCGCCAG//CACC------------------- 3' -14-4-20
5'CCT-----TCGTGCAGCTCCTAC//GACC------TCTAAAGACACCGCCAG//CACCTGAAGCATCCTCTACTCAG 3' -5-6
5'CCTCCACTTAAGTAGATGGATGCTTCCTGAGGAG------------------------GAAGCATCCTCTACTCAG 3' +25-386
5'CCTCCACTTCGTGCAGCTCCTAC+//GACCAC---CTCTAAAGACACCGCCAG//ACA------------------- 3' -3+3-920
5'--------TCGTGCAGCTCCTAC//GACCAC---CTCTAAAGACACCGCCAG//CACCTGAAGCATCCTCTACTCAG 3' -134-3
5'CCTCCACTTCGTGCAGCTCCTAC//GACC------TCTAAAGACACCGCCAG//CACCTGAAGCATCCTCTACTCAG 3' -6
5'CC--------------------//GACCACTGCCTCTAAAGACACCGCCAG//CACCT-------CCTCTACTCAG 3' -82-7-433
```

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Figure 9 CRISPR/Cas9 induced mutations in the toll/interleukin 1 receptor domain-containing adapter molecule (TICAM1) gene of channel catfish (*Ictalurus punctatus*).Blue sequence represents the protospacer adjacent motif (PAM) while the green sequence represents the target for gRNA. Indels induced by the simultaneous microinjection of three gRNAs are illustrated. Deletion mutations ranged from few base pair to more than 900 bp deletion and are represented by a dashed line in which each dash corresponds to a nucleotide that has been deleted. Red sequences are insertions. Double slash is present where a DNA sequence between two gRNA targets has been omitted for simplicity. When double slash is absent, this means that there is a large deletion and the entire sequence between 2 gRNA targets has been deleted. Each sequence starting with 5' and ending with 3' came from a single reaction representing a single allele. Mutations are reported in order starting with those at the 5' end e.g. +2-128-39 means 2 bp insertion, 128 bp deletion and 39 bp deletion.

3.1.2. RBL:

Mutations caused by the injection of RBL gRNAs no 4 and 5 were detected by PCR and surveyor® mutation detection assay. The two gRNAs induced large deletion detected by PCR as demonstrated in Figure 10. In some samples, such as sample no. 3 and 6 in Figure 10, there was no amplification of the wild type band (645 bp) suggesting that Cas9 protein cleaved the target sequence early in development, possibly at the one cell stage, resulting in mutations in all cells (Figure 10). Surveyor® assay was used to identify mutant samples that did not have the large deletions as shown in Figure 11. Mutated samples had additional band(s) not present in the wild type control (Figure 11).
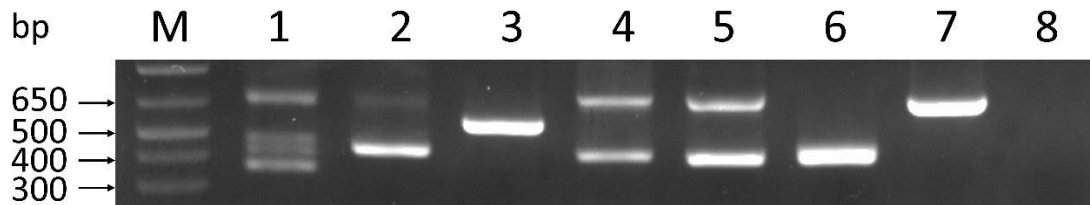
Figure 10 PCR amplification of a partial segment of channel catfish (*Ictalurus punctatus*) rhamnose binding lectin (RBL) gene using RBL F (forward) and RBL R (reverse) primers. Samples 1-6 came from embryos that were microinjected with gRNA/Cas9 protein while sample 7 represents wild type channel catfish that was full sib to embryos 1-6. Mutated embryos had large deletion demonstrated by the short band(s) compared to the wild type 645 bp band. Lane M contained 1 kb plus DNA ladder (Invitrogen) while lane 8 represents a no-DNA control.
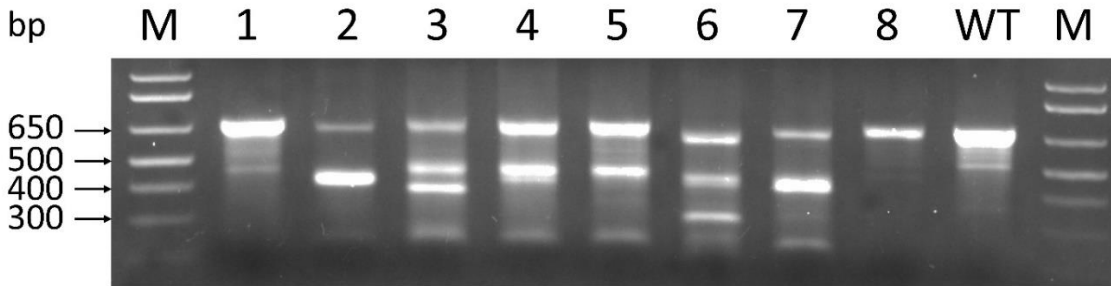
Figure 11 Surveyor® digestion of a partial segment of channel catfish (*Ictalurus punctatus*) rhamnose binding lectin (RBL) gene amplified with RBL F (forward) and RBL R (reverse) primers. Samples 1-8 came from embryos that were microinjected with gRNA/Cas9 protein. Samples were compared to the full sib wild type (wt) channel catfish control. Samples 2-7 were mutated while samples 1 and 8 were not mutated. Lanes M contained 1 kb plus DNA ladder (Invitrogen).

Effects of microinjection of different dosages of RBL gRNAs on the mutation rate were similar to TICAM 1 (Table 8). There was a positive correlation between the gRNA/Cas9 protein dosage and the mutation rate ($r = 0.542$, n = 17, $p = 0.025$). Except for the high dosage in which dead embryos and 4-month old fingerlings had the same mutation rate, mutation rate for other dosages was higher in dead embryos than 4-month old fry (Table 8, Figure 12) but the correlation between mutation rate and status (dead embryos or alive fingerlings) was not significant ($r = 0.263$, n = 17, $p = 0.308$).

DNA sequencing (Figure 13) confirmed the presence of mutation which ranged from a few bp to more than 300 bp deletion and few to several base pair insertions.

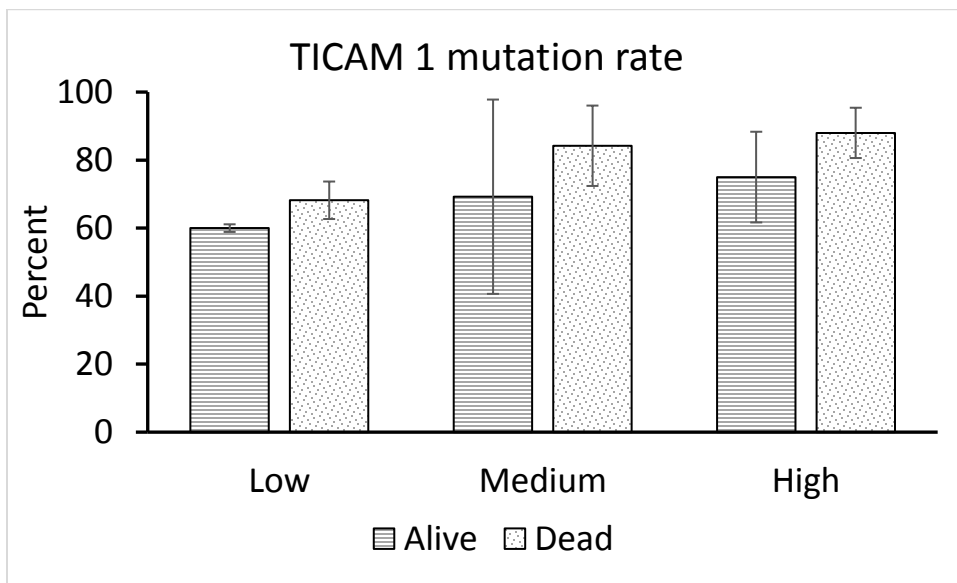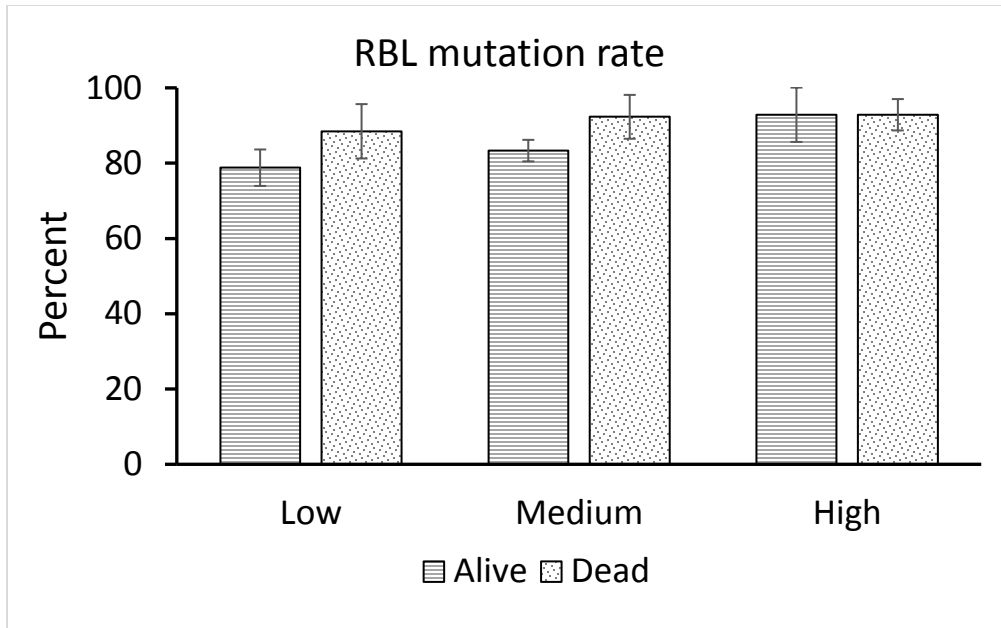Figure 12 Mutation rate ± standard error (SE) of channel catfish (*Ictalurus punctatus*) dead embryos and alive fingerlings microinjected with gRNA/Cas9 protein targeting rhamnose binding lectin (RBL) gene. Three dosages were microinjected into one-cell embryos (low dosage: 2.5 ng gRNA/7.5 ng Cas9, medium dosage: 5 ng gRNA/15 ng Cas9 and high dosage: 7.5 ng gRNA/22.5 ng Cas9).

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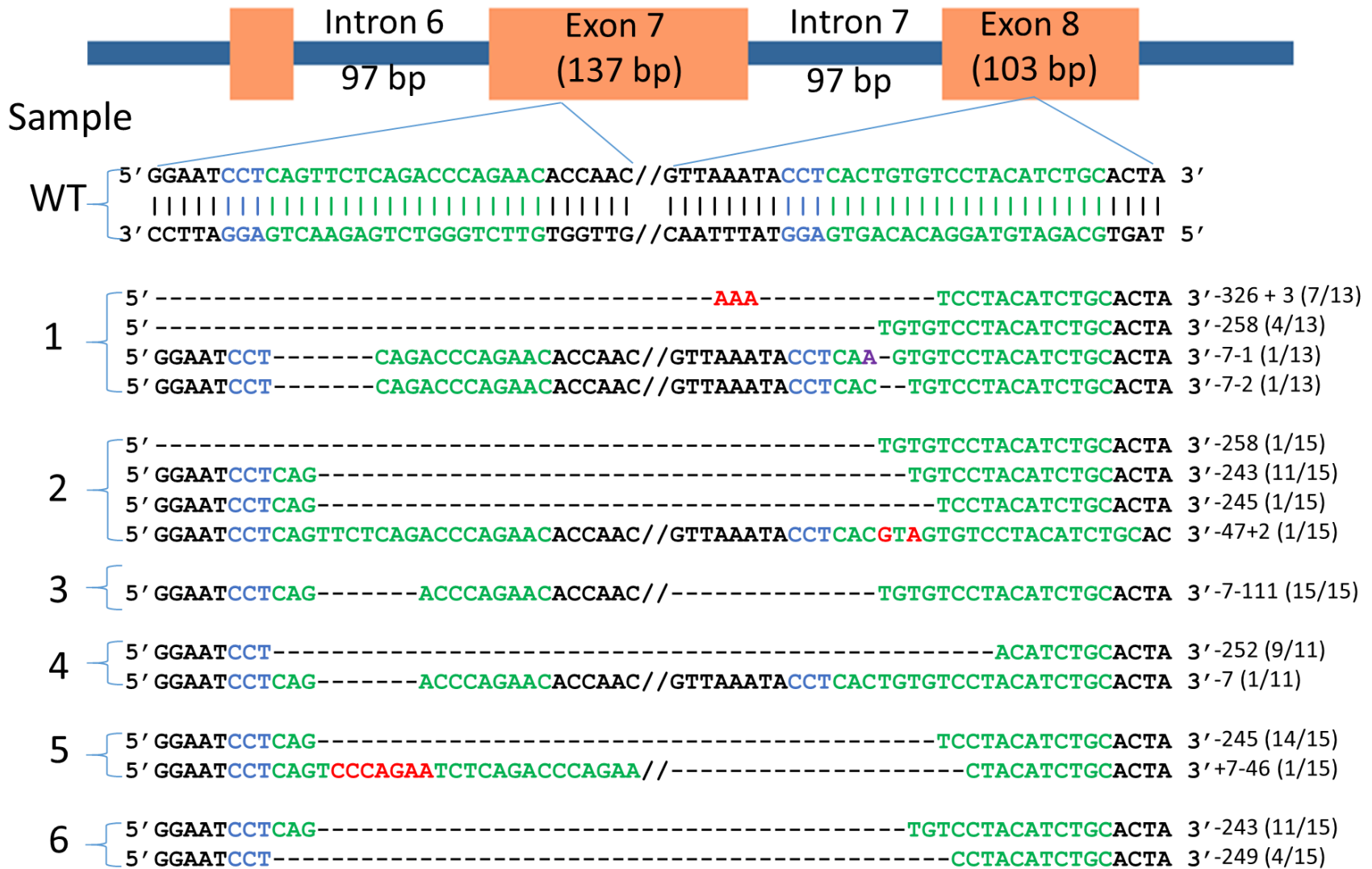
Figure 13 CRISPR/Cas9 induced mutations in rhamnose binding lectin (RBL) gene of channel catfish (*Ictalurus punctatus*). Blue sequence represents the protospacer adjacent motif (PAM) while the green sequence represents the target for gRNA. Indels induced by the simultaneous microinjection of 2 gRNAs are illustrated. Samples 1 and 2 were from the low dosage, 3 and 4 from the medium dosage while 5 and 6 from the high dosage. Deletion mutations ranged from few base pair to more than 300 bp deletion and are represented by a dashed line in which each dash corresponds to a nucleotide that has been deleted. Red sequences are insertions. Double slash is present where a DNA sequence between the two gRNA targets has been omitted for simplicity. When double slash is absent, this means that there is a large deletion and the entire sequence between 2 gRNA targets has been deleted. Each sequence starting with 5' and ending with 3' came from a single reaction representing a single allele. Mutations are reported in order starting with those at the 5' end e.g. -7-1 means 7 bp deletion and 1 bp deletion. Sequencing reactions with the same type of mutation are represented by a single sequence with a fraction of total e.g. (7/13) means that a certain type of mutation is present in 7 sequencing reactions out of 13.

Table 8 Total analyzed, number of mutated individuals and mean mutation rate of channel catfish (*Ictalurus punctatus*) dead embryos and alive fingerlings microinjected at one-cell stage with three dosages of gRNA/Cas9 protein targeting toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene. The three gRNA/Cas9 protein dosages were: low dosage: 2.5 ng gRNA/7.5 ng Cas9, medium dosage: 5 ng gRNA/15 ng Cas9 and high dosage: 7.5 ng gRNA/22.5 ng Cas9.

Treatment	Dead embryos			Alive fingerlings		
	Total analyzed	Mutated	Mean mutation % ± SE	Total analyzed	Mutated	Mean mutation % ± SE
TICAM 1[*]						
Low	22	15	68.2 ± 5.51	50	30	60.0 ± 1.12
Medium	19	16	84.2 ± 11.81	13	9	69.2 ± 28.57
High	25	22	88.0 ± 7.39	16	12	75.0 ± 13.33
RBL[**]						
Low	26	23	88.5 ± 7.22	33	26	78.8 ± 4.84
Medium	26	24	92.3 ± 5.82	12	10	83.3 ± 2.86
High	28	26	92.9 ± 4.17	14	13	92.9 ± 4.17
TICAM 1 and RBL combined[***]						
Low	48	38	79.2 ± 5.35	83	56	67.5 ± 4.96
Medium	45	40	88.9 ± 6.17	25	19	76.0 ± 12.18
High	53	48	90.6 ± 4.08	30	25	83.3 ± 7.76

[*]Positive correlation between gRNA/Cas9 dosage and mutation rate ($p = 0.084$).
[**]Positive correlation between gRNA/Cas9 dosage and mutation rate ($p = 0.025$).
[***]Positive correlation between gRNA/Cas9 dosage and mutation rate ($p = 0.013$)

3.1.3. Combined analysis for TICAM 1 and RBL mutation rate:

The same conclusions were obtained when the mutation data from TICAM 1 and RBL genes were combined (Table 8). Increasing the gRNA/Cas9 protein dosage increased the

mutation rate. There was a positive correlation between the gRNA/Cas9 protein dosage and the mutation rate ($r = 0.429$, n = 33, $p = 0.013$). Although the mutation rate was higher in dead embryos compared to alive fingerlings, no significant correlation was detected ($r = 0.245$, n = 33, $p = 0.153$).

Congenital anomalies

Congenital anomalies noticed in 4-month-old fingerlings included uni- or bilateral absence of eye development, unilateral absence of barbels, spinal and head deformities (Figure 14). All injected and non-injected 4-month-old control fry were free of any anomalies. Number, percent and genotype of fish with each type of anomaly are listed in Table 9. Except for one fingerling with head deformity in high dosage treatment of TICAM 1, all other fingerlings with congenital anomalies had mutated TICAM 1 and RBL genes.

Figure 14 Congenital anomalies in channel catfish (*Ictalurus punctatus*) 4-month-old fingerlings injected at the one cell stage with gRNAs/Cas9 protein targeting toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene. A: Absence of barbel development on the right side. B: The head is curved to the left side with deformities in the mouth. C: Spinal deformity. D: Absence of left eye development.

Table 9 Number and percent of channel catfish (*Ictalurus punctatus*) 4-month-old fingerlings

with congenital anomalies due to microinjection of three different dosages of gRNAs/Cas9

protein targeting toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1)

gene and rhamnose binding lectin (RBL) gene. Three dosages for each gene included low

dosage: 2.5 ng gRNA/7.5 ng Cas9 protein, medium dosage: 5 ng gRNA/15 ng Cas9 protein and

high dosage: 7.5 ng gRNA/22.5 ng Cas9 protein.

Gene	Dosage	Number and percent of fingerlings with anomaly*				Total number
		Absence of eye development	Absence of barbel	Deformed head	Spinal deformity	
TICAM	Low	0 (0%)	0 (0%)	0 (0%)	0 (0%)	50
	Medium	0 (0%)	0 (0%)	2 (15.4%)	0 (0%)	13
	High	0 (0%)	1 (6.3%)	3 (18.8%) (2 mutated)	0 (0%)	16
RBL	Low	2 (7.7%)	0 (0%)	1 (3.8%)	1 (3.8%)	26
	Medium	1 (10%)	0 (0%)	0 (0%)	0 (0%)	10
	High	1 (7.7%)	0 (0%)	0 (0%)	0 (0%)	13

*All fingerlings with anomalies were mutated except one from the TICAM 1 high dosage that
had head deformity but was not mutated.

3.2. Embryo mortality

3.2.1. TICAM 1

Embryo mortality started at 1 dpf. Mortality ranged from 23% in the nCTRL group to

82% in the high dosage group (Table 10). Plot of survival curves showed the similarity in

mortality between low dosage and iCTRL groups, and medium and high dosage groups (Figure

16). The lowest mean time to death for embryos was 4.2 and 4.3 dpf in the medium and high

dosage groups, respectively, while the highest was 7.4 days in the nCTRL group (Table 10).

Overall comparison of survival curves revealed significant differences in the mean survival time

to death ($p < 0.0001$) suggesting differences between at least 2 groups. Pairwise comparisons

detected significant differences in embryo mortality in all microinjected groups when compared

to the nCTRL group ($p < 0.0001$). Mortality in the low dosage group was not different from the

iCTRL group ($p = 0.295$). Mortality in the medium and high dosage groups was not different ($p

= 0.892$), however, it was significantly higher in the medium and high groups than all other

groups ($p < 0.0001$) (Table 10).
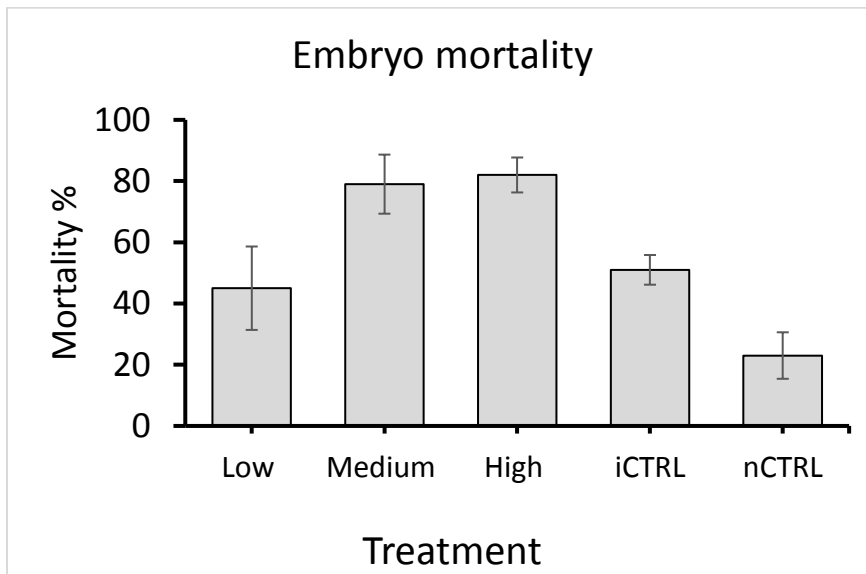
Figure 15 Embryo mortality of channel catfish (*Ictalurus punctatus*) embryos injected with

different dosages of gRNAs and Cas9 protein targeting toll/interleukin 1 receptor domain-

containing adapter molecule (TICAM 1) gene and two control groups (injected control, iCTRL,

and non-injected control, nCTRL). Guide RNA/Cas9 protein dosages included low dosage: 2.5

ng gRNA/7.5 ng Cas9 protein, medium dosage: 5 ng gRNA/15 ng Cas9 protein and high dosage:

7.5 ng gRNA/22.5 ng Cas9 protein. Medium and high dosages had the highest mortality % while

the non-injected group had the lowest mortality %. Low dosage had similar mortality to the

iCTRL.
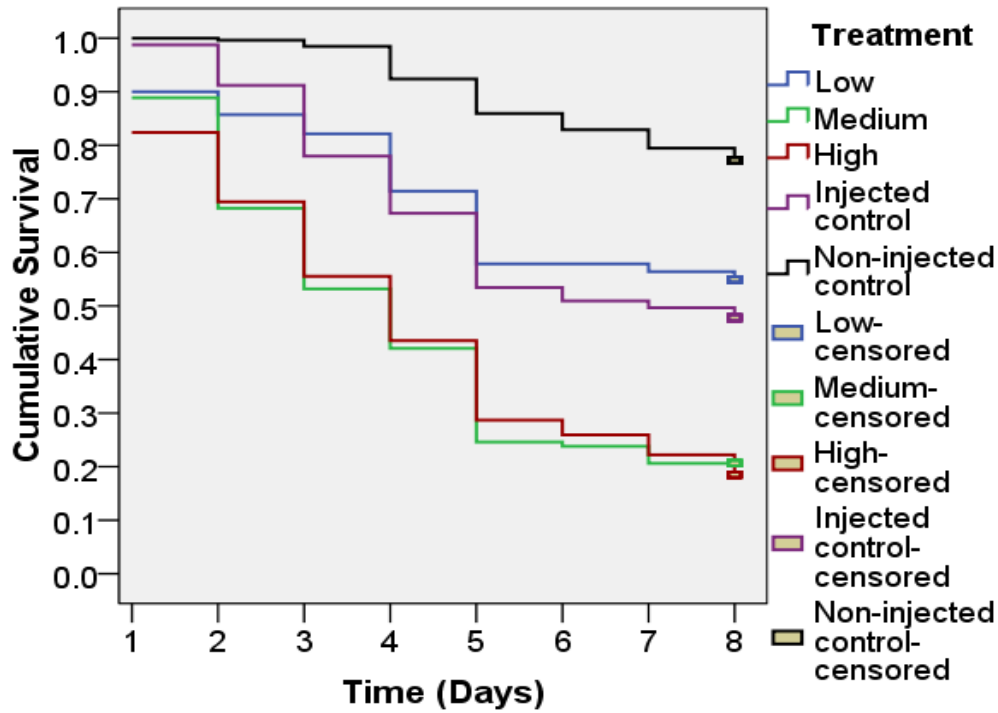
Figure 16 Kaplan-Meier plot of survival curves of channel catfish (*Ictalurus punctatus*) dead and

alive (censored) embryos injected with different dosages of gRNAs and Cas9 protein (low

dosage: 2.5 ng gRNA/7.5 ng Cas9 protein, medium dosage: 5 ng gRNA/15 ng Cas9 protein and

high dosage: 7.5 ng gRNA/22.5 ng Cas9 protein) targeting toll/interleukin 1 receptor domain-

containing adapter molecule (TICAM 1) gene and two control groups (injected control, iCTRL,

and non-injected control, nCTRL). Mortality in the low dosage group was not different from the

iCTRL group ($p = 0.295$). Mortality in the medium and high dosage groups was not different (p

$= 0.892$). All other pairwise comparisons were significantly different ($p < 0.05$).

3.2.2. RBL

Mortality started at 1 dpf and continued until 7 dpf when all embryos hatched. The lowest mortality percent was recorded in the nCTRL group (13.1 %) followed by the iCTRL group (29.8 %). The three treatment groups injected with gRNA/Cas9 protein had higher mortality (73.2-83.9 %) (Figure 17). One-way ANOVA revealed significant differences in the embryo mortality percent among different groups ($p < 0.0001$). In pairwise comparisons, significant differences were detected between nCTRL and all three treatment groups ($p < 0.0001$), iCTRL and all three treatment groups ($p < 0.0001$), iCTRL and nCTRL groups ($p = 0.002$) and low and medium dosage groups ($p = 0.044$). Comparison of mortality % between low and high dosages approached the significance level, however, it was not significant ($p = 0.064$). No significant differences in embryo mortality percent were detected between the medium and high dosage groups ($p = 0.999$).

The embryo mean time to death ranged from 4.2 to 6.7 days with nCTRL group having the longest time to death (Table 10). Survival curves were plotted (Figure 17) and overall comparison of survival curves revealed significant differences in mean time to death between at least two groups ($p < 0.0001$). With pairwise comparisons, the nCTRL group had significantly longer mean time to death than the iCTRL group ($p < 0.0001$) and the two control groups (iCTRL and nCTRL) had significantly longer mean time to death ($p < 0.0001$) than the three treatment groups (low, medium, high dosages). Embryo mean time to death in the medium dosage group was shorter but not significant when compared to the low dosage group ($p = 0.051$) and the high dosage group ($p = 0.098$). Low and high dosage groups had statistically similar embryo mean time to death ($p = 0.608$).

Figure 17 Mortality and survival of channel catfish, *Ictalurus punctatus*, embryos microinjected with different dosages of gRNA/Cas9 protein (low dosage: 2.5 ng gRNA/7.5 ng Cas9 protein, medium dosage: 5 ng gRNA/15 ng Cas9 protein and high dosage: 7.5 ng gRNA/22.5 ng Cas9 protein) targeting rhamnose binding lectin (RBL) gene compared to the injected control (iCTRL) and non-injected control (nCTRL) embryos. A: Mortality percent in the three treatment and two control groups. B: Kaplan-Meier plot of survival curves for the five groups with alive embryos being censored.

3.3. Embryo hatch

3.3.1. TICAM 1

Mean time to hatch ranged from 7.0 to 7.4 dpf for all the groups (Table 10). No significant differences were detected in the time to hatch for all the five groups ($p = 0.130$) (Figure 18). Non-injected control had the highest hatch percent (78%) while the high dosage group had the lowest hatch rate (18%) in all three replicates combined (Figure 18). One-way ANOVA revealed significant differences in the hatching percent between at least two groups (*F*

= 7.677, p = 0.004). Pairwise comparisons detected significant differences in the hatching

percent between the medium dosage and nCTRL (p = 0.009), high dosage and nCTRL (p =

0.005). Hatching percent in the low and high dosages approached significance (p = 0.09),

however, low and high dosages were not significantly different. No differences were detected in

all other pairwise comparisons (p > 0.05, Table 10).
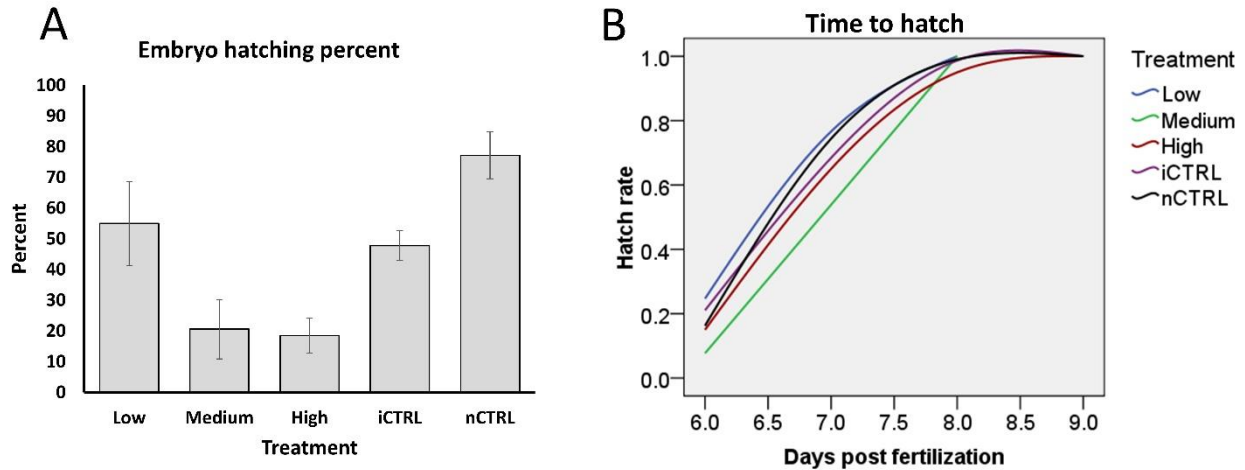
Figure 18 Embryo hatching of channel catfish (*Ictalurus punctatus*) embryos injected with

different dosages of gRNAs and Cas9 protein (low dosage: 2.5 ng gRNA/7.5 ng Cas9 protein,

medium dosage: 5 ng gRNA/15 ng Cas9 protein and high dosage: 7.5 ng gRNA/22.5 ng Cas9

protein) targeting toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1)

gene. A: Hatching percent in the three treatment groups (low, medium and high dosages) and

two control groups (injected control, iCTRL, and non-injected control, nCTRL). Medium and

high injection dosages had the lowest hatching % (p < 0.05) while the non-injected group had the

highest hatching %. B: Plot of time-to-hatch curves of the five groups. Hatching rate was

calculated as the fraction of total embryos that hatched.

3.3.2. RBL

Few individuals in the control groups started to hatch on 5 dpf while most of the fry hatched on 6 dpf. In the treatment groups, fry hatched on 6 and 7 dpf. All fry hatch was completed in all groups on 7 dpf. The highest hatching percent was recorded in the nCTRL group (86.9%) while the lowest hatch percentages were recorded in the medium and high dosage groups (16.1 and 16.2 % respectively) (Table 10, Figure 19). Comparison of mean hatching percent revealed significant differences between at least two groups ($p < 0.0001$). With pairwise comparisons, the following means for hatching percent were different: low and medium dosage groups ($p = 0.033$), low and high dosage groups ($p = 0.038$), iCTRL and the three treatment groups ($p < 0.0001$), nCTRL and the three treatment groups ($p < 0.0001$) and nCTRL and iCTRL groups ($p = 0.003$). Medium and high dosages had similar means for hatching percent ($p = 1.000$).

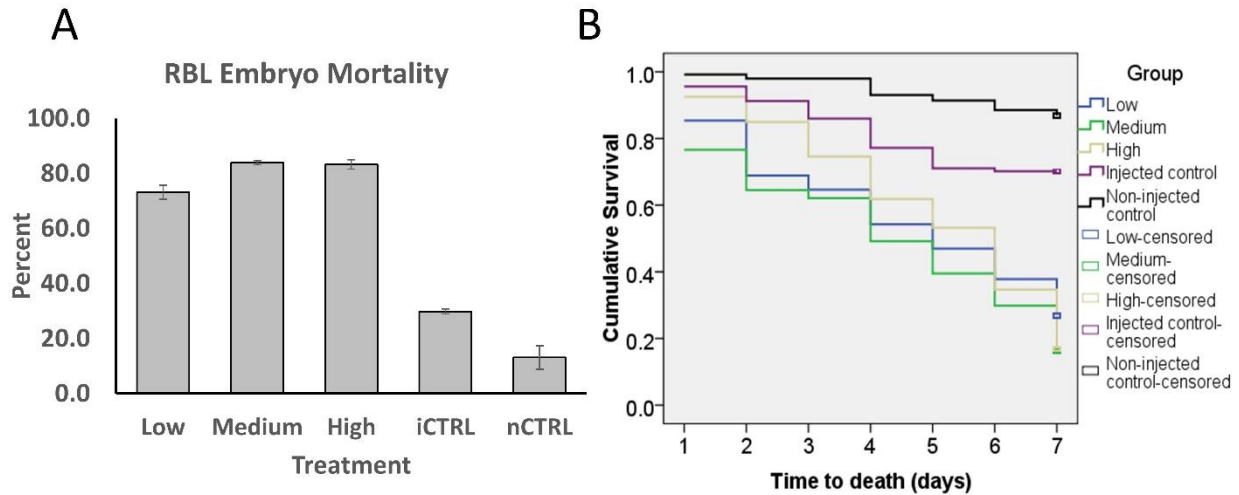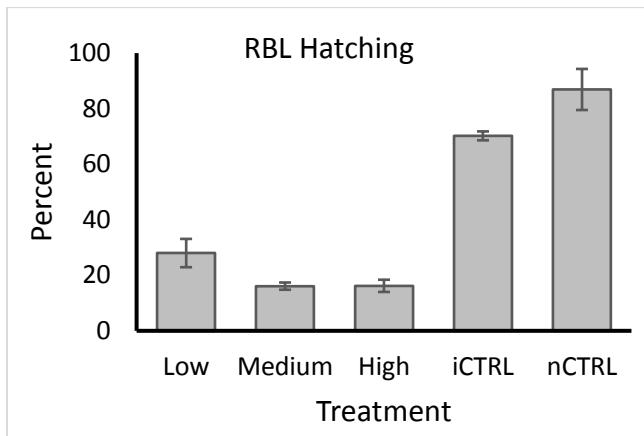Figure 19 Hatching percent of channel catfish, *Ictalurus punctatus*, embryos microinjected with different dosages of gRNA/Cas9 protein (low dosage: 2.5 ng gRNA/7.5 ng Cas9 protein, medium dosage: 5 ng gRNA/15 ng Cas9 protein and high dosage: 7.5 ng gRNA/22.5 ng Cas9 protein) targeting rhamnose binding lectin (RBL) gene compared to the injected control (iCTRL) and non-injected control (nCTRL) embryos.

Overall comparison of mean time to hatch revealed differences between groups ($p <$ 0.0001, Table 10). With pairwise comparisons, the means for time to hatch for the following groups were different: nCTRL and iCTRL hatched earlier than other three treatment groups ($p <$ 0.0001). Low dosage group hatched earlier than high dosage group ($p = 0.039$). Low and medium groups had similar mean time to hatch ($p = 0.184$) as well as medium and high groups ($p = 0.628$).

3.4. Early fry survival

3.4.1. TICAM 1

Fry started to die one day after hatch. Survival percent ranged from 54 to 97 % in medium dosage and nCTRL groups respectively (Figure 20, Table 10). Comparison of mean survival percent revealed significant differences between at least two groups (Welch's test, $p = 0.001$). Games-Howell pairwise comparisons detected significant differences in the survival percent between the iCTRL and nCTRL groups ($p = 0.001$). All other pairwise comparisons were not significant ($p > 0.05$). Accounting for the survival time to death, the nCTRL group had the highest mean survival time to death (17.7 dpf) while the medium dosage group had the shortest mean survival time to death (14.3 dpf) (Table 10). Survival curves were plotted (Figure 20) and pairwise comparisons of survival curves revealed significant differences between the nCTRL and iCTRL group ($p = 0.001$), nCTRL and the three treatment groups (low, medium, high dosages) ($p < 0.0001$). All other pairwise comparisons were not significant ($p > 0.05$).
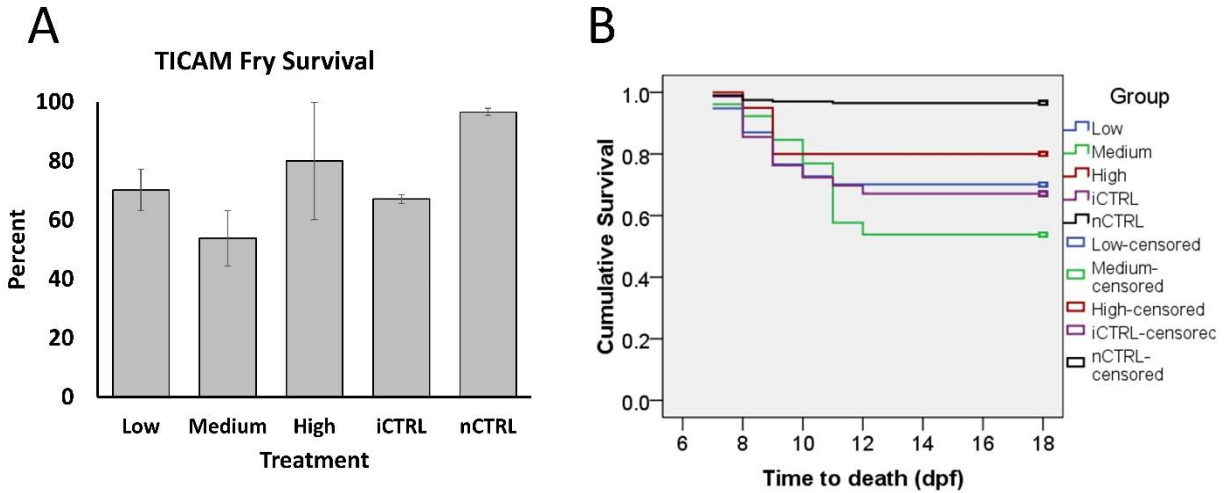
Figure 20 Early survival of channel catfish (*Ictalurus punctatus*) fry injected at the one-cell stage with different dosages of gRNAs and Cas9 protein (low dosage: 2.5 ng gRNA/7.5 ng Cas9 protein, medium dosage: 5 ng gRNA/15 ng Cas9 protein and high dosage: 7.5 ng gRNA/22.5 ng Cas9 protein) targeting toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene. A: The fry survival percent in the three treatment and two control groups at 18 day post fertilization (dpf). B: Kaplan-Meier plot of survival curves for the five groups with alive fry being censored.

3.4.2. RBL

The fry mean survival ranged from 62.1 to 97.5 % with the means of treatment groups lower than the control groups (Table 10) (Figure 21). The shortest mean survival time to death was recorded in the high dosage group (14.8 dpf) while the longest mean time to death was 17.8 dpf for the iCTRL group. Kaplan-Meier plot of survival curves (Figure 21) illustrated the differences in survival time to death between different groups. Overall comparison revealed differences between at least two groups ($p < 0.0001$). Pairwise comparisons detected the significant differences between control and treatment groups in mean time to death. nCTRL and iCTRL groups had similar fry mean time to death ($p = 0.170$). iCTRL group had significantly

longer mean time to death when compared to low dosage ($p = 0.001$), medium dosage and high

dosage groups ($p < 0.0001$). nCTRL group had significantly longer time to death when compared

to the low dosage ($p = 0.004$), medium dosage ($p = 0.003$) and high dosage groups ($p < 0.0001$).

Treatment groups had statistically similar mean survival times to death (low and medium $p =$

0.776, low and high $p = 0.107$, medium and high $p = 0.272$).
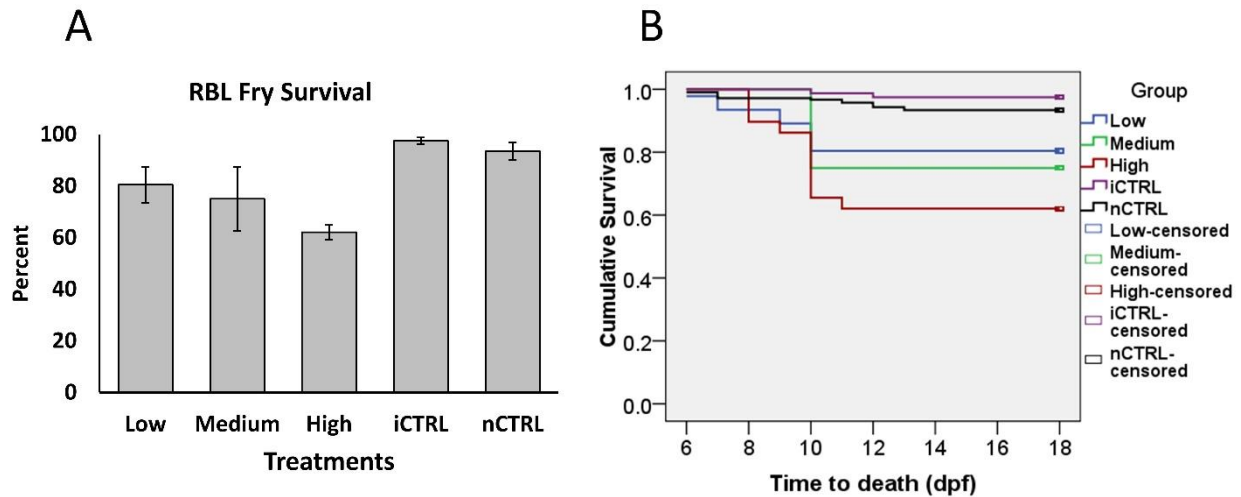
Figure 21 Early survival of channel catfish, *Ictalurus punctatus*, fry microinjected at the one-cell

stage with different dosages of gRNA/Cas9 protein (low dosage: 2.5 ng gRNA/7.5 ng Cas9

protein, medium dosage: 5 ng gRNA/15 ng Cas9 protein and high dosage: 7.5 ng gRNA/22.5 ng

Cas9 protein) targeting rhamnose binding lectin (RBL) gene compared to the injected control

(iCTRL) and non-injected control (nCTRL) groups. A: Mean percent survival of fry at 18 dpf. B:

Kaplan-Meier plot of survival curves for the five groups with alive fry being censored.

3.5. Pooled analysis (TICAM and RBL combined)

3.5.1. Embryo mortality

Embryo mortality percent ranged from 18.1% in the nCTRL group to 82.6% in the high

dosage group (Figure 22). Overall comparison of mortality % revealed significant differences (*p*

= 0.0001) between groups. Significant differences in mortality percent were detected between low and medium dosages ($p = 0.025$), low and high dosages ($p = 0.006$), low dosage and nCTRL (p = 0.006), medium dosage and both iCTRL and nCTRL ($p = 0.004$), high dosage and iCTRL ($p = 0.004$), high dosage and nCTRL ($p = 0.004$) and iCTRL and nCTRL ($p = 0.016$). No differences in mortality percent were detected between medium and high dosages ($p = 0.749$) and low dosage and iCTRL group ($p = 0.150$) (Table 10).

Figure 22 Embryo mortality of channel catfish (*Ictalurus punctatus*) embryos injected with different dosages of gRNAs and Cas9 protein targeting toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene compared to two control groups (injected control, iCTRL, and non-injected control, nCTRL). Guide RNA/Cas9 protein dosages included low dosage: 2.5 ng gRNA/7.5 ng Cas9, medium dosage: 5 ng gRNA/15 ng Cas9 and high dosage: 7.5 ng gRNA/22.5 ng Cas9. Medium and high dosages had the highest mortality % while the non-injected group had the lowest mortality %.

Kaplan-Meier test detected significant differences between at least two groups (p < 0.0001). Except medium and high dosage which had statistically similar mean time to death (p = 0.150), all other pairwise comparisons were different (p < 0.0001). nCTRL had the longest mean time to death (7.5 dpf) (p < 0.0001), followed by iCTRL which had the second longest mean time to death (6.2 dpf) (p < 0.0001). Mean time to death for low dosage group (5.4 dpf) was shorter than nCTRL and iCTRL groups (p < 0.0001) and longer than the medium and high dosage groups (p < 0.0001) which had a mean survival time of 4.3 and 4.8 dpf respectively (Figure 23, Table 10).
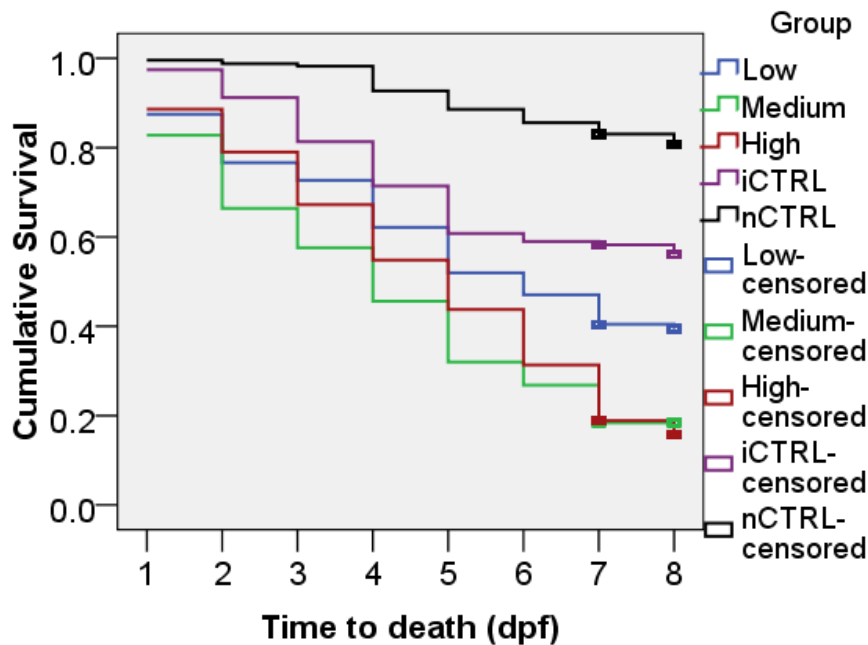
Figure 23 Kaplan-Meier plot of survival curves of channel catfish (*Ictalurus punctatus*) dead and alive (censored) embryos injected with different dosages of gRNAs and Cas9 protein targeting toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene compared to two control groups (injected control, iCTRL, and non-injected control, nCTRL). Three dosages were injected (low dosage: 2.5 ng gRNA/7.5 ng Cas9,

medium dosage: 5 ng gRNA/15 ng Cas9 and high dosage: 7.5 ng gRNA/22.5 ng Cas9). Mortality

in the low dosage group was not different from the iCTRL group ($p = 0.295$). Except for

mortality percent in the medium and high dosage groups, which was not different ($p > 0.05$), all

other pairwise comparisons were significantly different ($p < 0.05$).

3.5.2. Embryo hatch

Overall hatching percent was significantly different among the five groups ($p < 0.0001$)

(Table 7). Hatching % was statistically different in low and medium dosages ($p = 0.025$), low

and high dosages ($p = 0.006$), low and iCTRL ($p = 0.006$), low and nCTRL ($p = 0.004$), medium

and iCTRL (0.004), high dosage and iCTRL ($p = 0.004$), high and nCTRL ($p = 0.004$) and

iCTRL and nCTRL ($p = 0.016$). No differences in the hatching percent were detected between

medium and high dosages ($p = 0.749$) and low and iCTRL ($p = 0.150$) (Figure 24, Table 10).
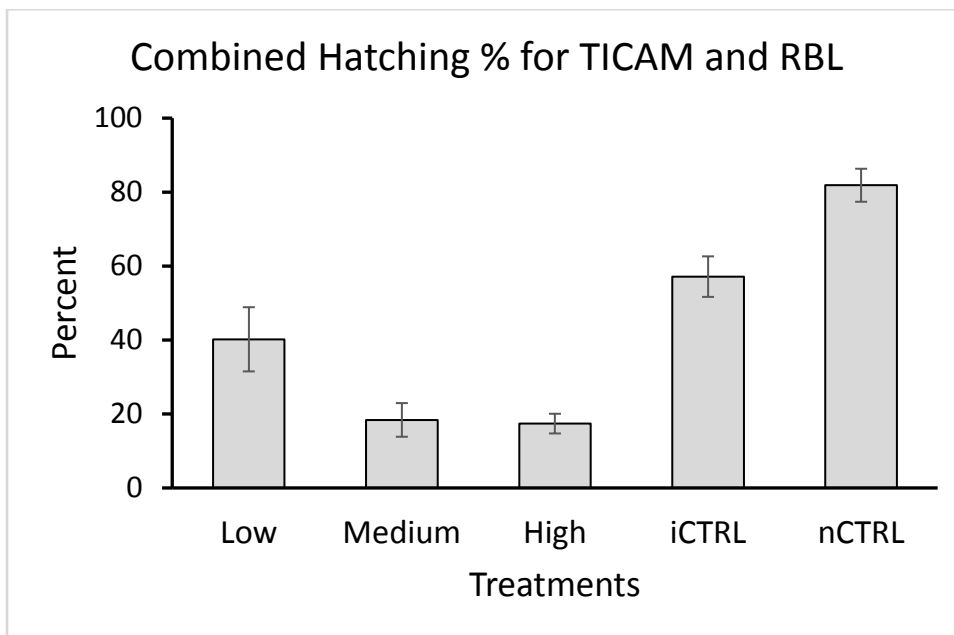
Figure 24 Hatching percent of channel catfish (*Ictalurus punctatus*) embryos injected with

different dosages of gRNAs and Cas9 protein targeting toll/interleukin 1 receptor domain-

containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene

combined. Three dosages were injected (low dosage: 2.5 ng gRNA/7.5 ng Cas9, medium dosage: 5 ng gRNA/15 ng Cas9 and high dosage: 7.5 ng gRNA/22.5 ng Cas9). Medium and high injection dosages had the lowest hatching % ($p < 0.05$) while the non-injected control (nCTRL) had the highest hatching %.

Time to hatch for each group was plotted (Figure 25) and comparison of mean time to hatch revealed significant differences between at least two groups ($p = 0.001$). Mean time to hatch was significant different between nCTRL and low dosage ($p = 0.022$), nCTRL and medium dosage ($p = 0.0004$), nCTRL and high dosage ($p = 0.016$), low and medium dosages ($p = 0.038$) and medium dosage and iCTRL ($p = 0.005$). All other pairwise comparisons of time to hatch were not significant ($p > 0.05$) (Table 10).

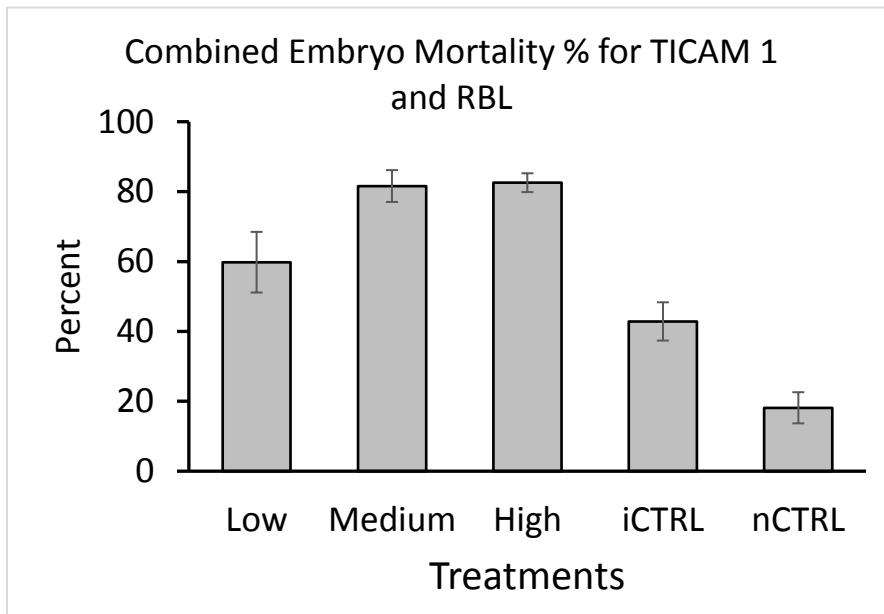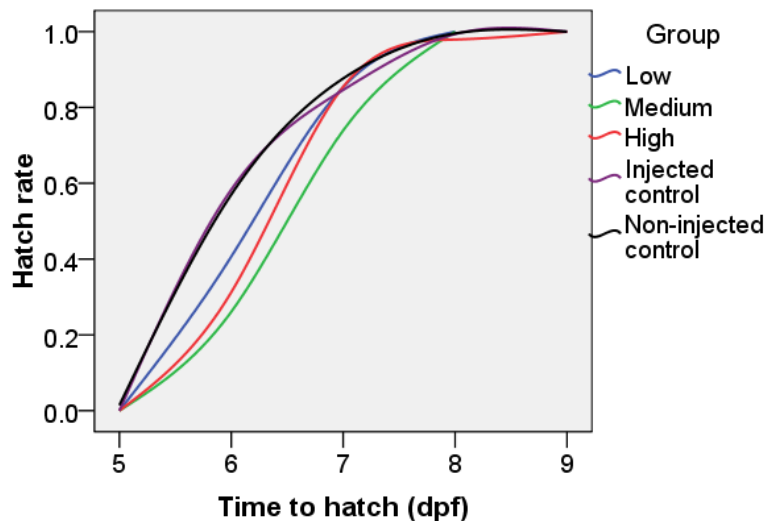Figure 25 Plot of time-to-hatch (days post fertilization, dpf) of channel catfish (*Ictalurus punctatus*) embryos injected with different dosages of gRNAs and Cas9 protein targeting toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene combined. Three dosages were injected (low dosage: 2.5 ng

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gRNA/7.5 ng Cas9, medium dosage: 5 ng gRNA/15 ng Cas9 and high dosage: 7.5 ng

gRNA/22.5 ng Cas9). Hatching rate was calculated as the fraction of total embryos that hatched.

3.5.3. Fry survival

The highest fry survival percent ± standard error was recorded in the nCTRL group

(94.9% ± 4.40) followed by the iCTRL group (82.7% ± 16.62) while the lowest was recorded in

the medium group (63.0 ± 18.97) (Figure 26), however, fry survival % was not significantly

different in all five groups ($p > 0.05$) (Table 10).
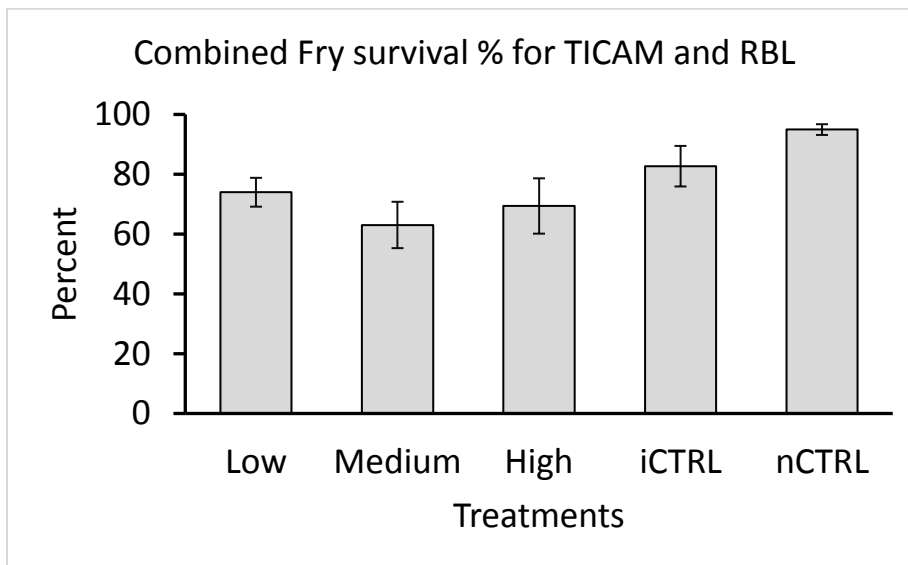
Figure 26 Combined fry survival percent of channel catfish (*Ictalurus punctatus*) injected with

different dosages of gRNAs and Cas9 protein targeting toll/interleukin1 receptor domain-

containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene. Three

dosages were injected (low dosage: 2.5 ng gRNA/7.5 ng Cas9, medium dosage: 5 ng gRNA/15

ng Cas9 and high dosage: 7.5 ng gRNA/22.5 ng Cas9). Survival % was calculated at 18 days

post fertilization. Medium and high injection dosages had the lowest hatching % ($p < 0.05$) while

the non-injected control (nCTRL) had the highest hatching %.

Kaplan-Meier test revealed significant differences in the fry mean time to death among groups ($p < 0.0001$). Survival curves were plotted (Figure 27) and the fry survival time was longer in the control groups than the treatment groups with the following pairwise comparisons being significant: the three treatment groups (low, medium and high) and nCTRL ($p < 0.0001$), medium and iCTRL ($p = 0.007$), high and iCTRL ($p = 0.048$) and iCTRL and nCTRL ($p < 0.0001$) (Table 7). All other pairwise comparisons were not significant ($p > 0.05$) (Table 7).
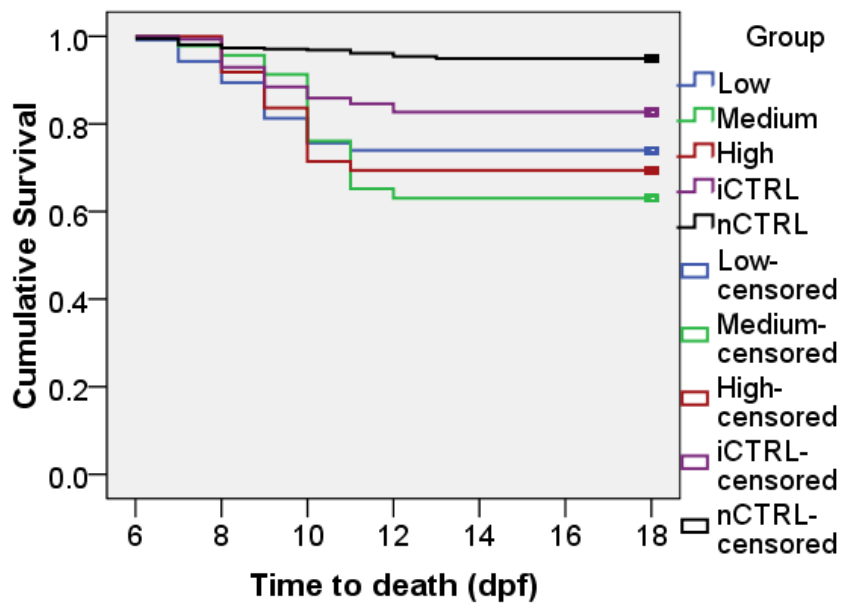
Figure 27 Kaplan-Meier plot of survival curves of channel catfish (*Ictalurus punctatus*) fry injected at the one-cell stage with different dosages of gRNAs and Cas9 protein (low dosage: 2.5 ng gRNA/7.5 ng Cas9, medium dosage targeting toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene. Three dosages were injected (5 ng gRNA/15 ng Cas9 and high dosage: 7.5 ng gRNA/22.5 ng Cas9). Survival was calculated starting at the fry hatch date until 18 days post fertilization (dpf) where alive fry were censored.

Table 10 Total injected embryos, number and percent of dead and alive embryos, mean time to death (days post fertilization, dpf) ± standard error (SE), mean time to hatch (dpf) ± SE, hatch % ± SE, fry survival % ± SE and fry mean survival time to death (dpf) ± SE for channel catfish, *Ictalurus punctatus*, embryos injected with different dosages of guide RNAs (gRNAs) and Cas9 protein targeting the channel catfish toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene. Three dosages of gRNA were injected including low dosage (2.5 ng gRNAs, 7.5 ng Cas9 protein), medium dosage (5 ng gRNAs, 15 ng Cas9 protein) and high dosage (7.5 ng gRNAs, 22.5 ng Cas9 protein). Two control groups included a control that has been injected with the same volume of injection solution (composition: water with up to one third of 0.5% phenol red solution, no gRNA/Cas9 protein) as the treatment groups which was 50 nanoliters for all microinjected embryos (iCTRL) and a non-injected control (nCTRL). Significant differences are marked with different superscript letters.

Group	Total embryos injected	Dead embryos		Alive embryos		Embryo mean time to death (dpf) ± SE	Mean time to hatch (dpf) ± SE	Hatch % ± SE	Fry survival % ± SE	Fry mean time to death (dpf) ± SE
		Number	%	Number	%					
TICAM 1										
Low	140	63	45.0	77	55.0	6.0 ± 0.22^a	7.0 ± 0.08^a	55.0 ± 13.63^{ab}	70.1 ± 6.99^{ab}	15.2 ± 0.49^a
Medium	126	100	79.4	26	20.6	4.2 ± 0.21^b	7.4 ± 0.13^a	20.6 ± 9.65^a	53.8 ± 9.35^{ab}	14.3 ± 0.81^a
High	108	88	81.5	20	18.5	4.3 ± 0.24^b	7.3 ± 0.18^a	18.5 ± 5.71^a	80.0 ± 19.90^{ab}	16.2 ± 0.83^a
iCTRL	159	83	52.2	76	47.8	5.9 ± 0.18^a	7.1 ± 0.10^a	47.8 ± 4.85^{ab}	67.1 ± 1.51^a	15.1 ± 0.49^a
nCTRL	263	60	22.8	203	77.2	7.4 ± 0.08	7.1 ± 0.05^a	77.2 ± 7.60^b	96.6 ± 1.31^b	17.7 ± 0.13

Table 10 *Continued*.

Group	Total embryos injected	Dead embryos		Alive embryos		Embryo mean time to death (dpf) ± SE	Mean time to hatch (dpf) ± SE	Hatch % ± SE	Fry survival % ± SE	Fry mean time to death (dpf) ± SE
		Number	%	Number	%					
RBL										
Low	166	120	72.2	46	27.7	4.6 ± 0.18^{a}	6.3 ± 0.07^{a}	27.7 ± 2.57	80.4 ± 7.06	16.2 ± 0.56^{a}
Medium	124	104	83.9	20	16.1	4.2 ± 0.22^{a}	6.5 ± 0.12^{ab}	16.1 ± 0.75^{a}	75.0 ± 12.37	16.0 ± 0.78^{a}
High	173	145	83.8	28	16.2	5.0 ± 0.15^{a}	6.6 ± 0.10^{b}	16.2 ± 1.27^{a}	62.1 ± 2.75	14.8 ± 0.78^{a}
iCTRL	114	34	29.8	80	70.2	5.9 ± 0.17	6.1 ± 0.03^{c}	70.2 ± 0.90	97.5 ± 1.27	17.8 ± 0.12^{b}
nCTRL	244	32	13.1	212	86.9	6.7 ± 0.07	6.1 ± 0.02^{c}	86.9 ± 4.25	93.4 ± 3.39	17.4 ± 0.15^{b}
TICAM and RBL combined										
Low	306	183	59.8^{b}	123	39.8	5.4 ± 0.15	6.7 ± 0.06^{b}	40.2 ± 8.68^{b}	74.0 ± 4.82^{a}	15.6 ± 0.37^{ab}
Medium	250	204	81.6^{a}	46	18.4	4.3 ± 0.16^{a}	7.0 ± 0.11^{a}	18.4 ± 4.55^{a}	63.0 ± 7.75^{a}	15.0 ± 0.58^{a}
High	281	232	82.6^{a}	49	17.4	4.8 ± 0.14^{a}	6.9 ± 0.10^{ab}	17.4 ± 2.66^{a}	69.4 ± 9.25^{a}	15.3 ± 0.58^{a}
iCTRL	273	117	42.9^{b}	156	57.1	6.2 ± 0.14	6.6 ± 0.06^{bc}	57.1 ± 5.48^{b}	82.7 ± 6.78^{a}	16.5 ± 0.27^{b}
nCTRL	507	92	18.1	415	81.9	7.5 ± 0.06	6.5 ± 0.04^{c}	81.9 ± 4.46	94.9 ± 1.80^{a}	17.6 ± 0.10

4. Discussion

In the current study, the effects of microinjection of three dosages of guide RNA (gRNA)/Cas9 protein (low, 2.5 ng gRNA/7.5 ng Cas9, medium, 5 ng gRNA/15 ng Cas9 and high, 7.5 ng gRNA/22.5 ng Cas9) on the mutation rate, embryo mortality, hatchability and early fry survival in channel catfish were investigated. Efficient mutagenesis was achieved as demonstrated by PCR, Surveyor® assay and DNA sequencing. The higher dosages achieved higher mutation rates. The mutation rate was higher for each dosage in 4-month-old fingerlings than dead embryos. Microinjection procedure increased the embryo mortality when injected control embryos were compared to non-injected controls. Injection of gRNA/Cas9 protein increased the embryo mortality and congenital anomalies in 4-month-old fingerlings when compared to the injected control embryos. Hatching percent was reduced with injection of higher dosages of gRNA/Cas9 protein.

Microinjection continues to be the most effective and reliable method to deliver biologically active substances such as DNA, RNA or protein into embryos (Xu, 1999), however, microinjected embryos had lower survival rates when compared to their non-injected siblings (Rosen et al., 2009, Schubert et al., 2014). Survival of microinjected embryos depends on the substance and the volume being injected. The larger the volume of microinjection, the higher the embryo mortality. (Schubert et al., 2014). Schubert el al. (2014) found that increasing the injection volume increased embryo mortality. In their study, injection of 0.5 nL autoclaved water reduced the survival of zebrafish embryos at 96 h from 98% to 95% while the injection of 4.2 nL reduced the survival to 84% when compared to controls (98%).

Ideal volumes injected into zebrafish (*Danio rerio*) embryos were variable depending on where injection took place. For delivery of injection materials into one cell embryo, 10 – 20% of the cytoplasmic volume was injected (Xu, 1999). For microinjection into the yolk, up to 10% of the egg volume can be injected (Rosen et al., 2009). In case of the fully automated robotic microinjection, 3 nL were injected into single zebrafish embryos (Wang et al., 2007) which is 3 times the volume injected by Yin et al. (2015). The injection of 3 nL (300 nM) of fluorescein-tagged morpholinos targeting the gene *no tail* into the cytoplasm of one cell zebrafish embryos with an automated microinjection system resulted in 98% survival with 98.5 of surviving embryos exhibiting the intended tailless phenotype (Wang et al., 2007). In this study, 50 nL were injected into the yolk which is about 0.04% of the total egg volume assuming the average diameter of channel catfish eggs is 3 mm (Chapman, 2000).

Part of the embryo mortality in this experiment could be attributed to the microinjection procedures. Comparing the embryo mortality in nCTRL with the iCTRL groups confirmed the adverse effects of microinjection on embryo survival. Embryo survival was reduced from 81.9% to 57.1% due to microinjection of 50 nL of buffer (water and phenol red). The nCTRL group had significantly longer mean time to death and higher survival percent when compared to the iCTRL group. Except for microinjection, embryos in the 2 groups were full sib and have been exposed to the same handling stress. The same result was obtained for both genes, TICAM 1 and RBL. A similar conclusion was drawn when zebrafish embryos were microinjected with 0.5 and 4.2 nL of autoclaved water (Schubert et al., 2014). In the current study, a coloring material (phenol red) was used to track the injection. This technique has been used by several researchers (Yuan and Sun, 2009, Benard et al., 2012, Feng et al., 2015, Hisano et al., 2015, Yin et al., 2015,

Erickson et al., 2016, Xiao and Zhang, 2016), however, the effects of adding phenol red to the injection material on survival rate of fish embryos have not been investigated.

Another cause of embryo mortality could be due to gRNA/Cas9 protein dose. To assess the effects of different doses of gRNA/Cas9 protein on embryo survival, a group of embryos were injected with the same injection volume without gRNA/Cas9 protein. The three treatments (low, medium and high dosage) and iCTRL groups were full sib, exposed to the same handling stress and reared in the same environmental conditions with three replicates for each treatment to reduce the experimental error. However, in the six gRNA/Cas9 treatments for TICAM 1 and RBL genes combined, five treatments (all except the low dose for TICAM1 gene) showed significant effects of gRNA/Cas9 protein where embryo survival was reduced when compared to the iCTRL treatment. In the current experiment, embryo hatching % varied depending on the treatment with the lowest hatch rate in high dosage (17.4 % for TICAM 1 and RBL combined) which is still higher than the hatch rate (9%) obtained by Oin, (2015) when channel catfish embryos were microinjected with 100 picogram of gRNA and 300 picogram of Cas9 mRNA targeting gonadotropin releasing hormone (GnRH). The volume of injection was not reported and no coloring material was used. In his injected control embryos, hatch rate was 11% which is less than the hatch rate of injected control embryos in the current study (57.1% for TICAM 1 and RBL combined).

Increasing the concentration of TICAM 1 gRNA/Cas9 protein increased the mutation rate of both genes as well as the mortality rate of channel catfish embryos. In zebrafish, a similar conclusion was obtained when varying concentrations of gRNA and Cas9 mRNA were injected (Shah et al., 2015). The researchers concluded that increasing the concentration of Cas9 mRNA

injected into zebrafish embryos led to increasing occurrence of toxic phenotypes ranging from death after several hours to general problems with the heart, nervous system and axis formation. When higher concentrations of gRNAs targeting the solute carrier family 24 member 5 (SLC24A5) gene were injected into zebrafish embryos, gene knockout as well as toxicity increased (Shah et al., 2015).

DNA sequencing proved the efficiency of CRISPR/Cas9 system in inducing mutations in channel catfish. The frequency of different types of mutations can give an idea about how early in the development CRISPR/Cas9 protein induced-mutations take place. The fewer types of mutations with high frequency may indicate cleavage of DNA targets by Cas9 protein at earlier developmental stages. In RBL, low dosage embryos had four types of mutations. In the medium dosage, there were two types of mutations in one mutated embryo while in another one, there was only one type of mutation in all sequencing reactions (15/15) suggesting the possibility of mutating RBL gene at the one-cell stage. No wild type alleles were detected by sequencing in medium and high dosage. The two types of mutation in medium and high dosages suggest bi-allelic mutation of RBL gene in which DSB in each of the two alleles resulted in a different type of mutation. CRISPR/Cas9 induced bi-allelic mutations were reported in zebrafish when the tyrosinase gene was targeted (Jao et al., 2013). Tyrosinase mutant embryos were mosaic for pigmentation suggesting that there are still some cells with the wild type tyrosinase gene that can produce pigmentation.

TICAM 1 and RBL are immune-related genes that play a significant role in the disease progression pathway of enteric septicemia of catfish and columnaris disease, respectively (Baoprasertkul et al., 2006, Beck et al., 2012, Peatman et al., 2013, Thongda et al., 2014).

However, there is not enough information on their pleiotropic effects and if knockout of the 2 genes would affect survival. Successful knockout of those genes has been achieved as described in chapters 2 and 3, 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 (Ho et al., 1997, Söker et al., 2008) such as the immune suppression in myostatin-deficient medaka (Chiang et al., 2015). The possibility of the effects of off-target mutations on embryo survival exists. However, many researchers reported that low off-target frequency is associated with CRISPR/Cas9 system (Cho et al., 2014, Smith et al., 2014, Veres et al., 2014, Kim et al., 2015). In contrast, some researchers reported substantial off-target effects for the CRISPR/Cas9 system (Cradick et al., 2013, Fu et al., 2013, Pattanayak et al., 2013). These conflicting conclusions might be due to the design of gRNA, the method of off-target mutation detection or the gene being targeted (Kim et al., 2015). Toxic effects of gRNA depend on the genes that may have been off-targeted and it is difficult to eliminate this possibility in the current experiment. In RBL, the three doses of gRNA resulted in statistically similar embryo survival that was different from the iCTRL treatment. However, with more replication, the low dose may result in statistically lower mortality as the embryo survival was 18% higher than the closest (high) dose.

The introduction of nucleic acids into fish embryos may affect the embryo hatch rate and time (Dunham, 2011, Su et al., 2015). In channel catfish, these effects are usually reduced hatch rate and extended hatching time of transgenic embryos when compared to controls (Su et al., 2015). In the current experiment, the effects of different doses of gRNA/Cas9 protein on the mean time to hatch were variable. In TICAM 1, no differences in mean time to hatch were detected between the treatments and controls suggesting that, under the current experimental

conditions, neither microinjection procedures nor different doses of gRNA/Cas9 protein have significant effects on hatch time. In RBL, microinjection procedures did not affect the time to hatch (nCTRL and iCTRL groups had similar mean time to hatch). However, gRNA/Cas9 injection delayed the hatch time when compared to controls. Moreover, increasing the dose of gRNA/Cas9 increased the time to hatch (delayed hatch). Su et al. (2015) found that the effects of transgenic sterilization constructs introduced by electroporation on embryo hatching time were variable. Some constructs did not affect the mean time to hatch while others delayed the embryo hatch time when compared to controls.

The effects of gRNA/Cas9 on delaying the embryonic development and subsequent extension of embryo time to hatch cannot be eliminated. Those effects may be attributed to the double strand breaks induced in the DNA. The adverse effects of DNA damage on embryonic development exist and such effects depend on the severity of the DNA damage. In mice, severe sperm DNA damage induced by sperm chromatin fragmentation (SCF) resulted in delayed paternal DNA replication and retarded the embryonic development and a large proportion of the embryos were arrested at the G2/M (second gap phase/mitosis) border (Gawecka et al., 2013). There is also time needed by the cell to repair the DNA double strand break which may delay embryonic development and result in temporary cell arrest during the cell cycle until the DNA repair is complete (Lliakis, 1991). This time ranges from several minutes to several hours depending on the type of cells, the repair mechanism and the severity of the DNA double strand breaks (Bradley and Kohn, 1979, Iliakis et al., 1991, Nunez et al., 1995).

Microinjection procedure decreased the hatching percent significantly in RBL, while in TICAM 1, the hatch percent in the nCTRL group was still 22% higher than iCTRL group, but

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the difference was not statistically different due to the high variation in hatch percent between replicates. The injection of gRNA/Cas9 protein reduced the hatch % significantly in RBL when compared to the iCTRL group, and increasing the dosage from low to medium reduced the hatch %, which is mainly affected by the embryo mortality in each treatment. Although it was not significant for TICAM 1, the hatch % was reduced by more than 30% when the dosage was increased from low to medium. Fry in most treatments had significantly lower mean survival time when compared to the nCTRL revealing the possible effects of microinjection and/or the dosage of gRNA/Cas9 on early fry survival. The microinjection procedure significantly reduced the early fry survival in TICAM 1 but gRNA/Cas9 protein did not affect fry survival. In RBL, the gRNA/Cas9 protein reduced fry survival.

5. Conclusion

The effects of microinjection of different dosages of gRNAs/Cas9 targeting TICAM 1 and RBL genes on embryo survival, hatch and early fry survival were evaluated. Microinjection procedures increased embryo mortality. Increasing the dosage of gRNA/Cas9 protein increased embryo mortality and reduced hatching percent. Effects on mean time to hatch were different depending on the gene being targeted. In TICAM 1, no differences were detected between treatment and control groups. In RBL, mean time to hatch was longer in treatment groups when compared to controls. Means of fry survival time to death were similar for the three dosages. The current study paves the way for better design of genome editing studies in catfish and can be modified for other fish species. Determining the best dosage for mutation, embryo survival, hatch and fry survival would facilitate the generation of sufficient numbers of founder fish carrying different mutations.

6. References

Ablain, J., Durand, E.M., Yang, S., Zhou, Y. & Zon, L.I. (2015). A CRISPR/Cas9 vector system for tissue-specific gene disruption in zebrafish. *Developmental cell,* 32**:** 756-764.

Armstrong, J., Duhon, S. & Malacinski, G. (1989). Raising the axolotl in captivity. *Developmental biology of the axolotl***:** 220-227.

Auer, T.O., Duroure, K., De Cian, A., Concordet, J.-P. & Del Bene, F. (2014). Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. *Genome research,* 24**:** 142-153.

Baoprasertkul, P., Peatman, E., Somridhivej, B. & Liu, Z. (2006). Toll-like receptor 3 and TICAM genes in catfish: species-specific expression profiles following infection with *Edwardsiella ictaluri*. *Immunogenetics,* 58**:** 817-830.

Beck, B.H., Farmer, B.D., Straus, D.L., Li, C. & Peatman, E. (2012). Putative roles for a rhamnose binding lectin in *Flavobacterium columnare* pathogenesis in channel catfish *Ictalurus punctatus*. *Fish & shellfish immunology,* 33**:** 1008-1015.

Benard, E.L., Van Der Sar, A.M., Ellett, F., Lieschke, G.J., Spaink, H.P. & Meijer, A.H. (2012). Infection of zebrafish embryos with intracellular bacterial pathogens. *JoVE (Journal of Visualized Experiments)***:** e3781-e3781.

Bradley, M.O. & Kohn, K.W. (1979). X-ray induced DNA double strand break production and repair in mammalian cells as measured by neutral filter elution. *Nucleic acids research,* 7**:** 793-804.

Chapman, F.A. (2000). *Farm-raised channel catfish*: University of Florida Cooperative Extension Service, Institute of Food and Agriculture Sciences, EDIS.

Chiang, Y.-A., Kinoshita, M., Maekawa, S., Kulkarni, A., Lo, C.-F., Yoshiura, Y., Wang, H.-C. & Aoki, T. (2015). TALENs-mediated gene disruption of myostatin produces a larger phenotype of medaka with an apparently compromised immune system. *Fish & shellfish immunology*.

Chisada, S.-I., Okamoto, H., Taniguchi, Y., Kimori, Y., Toyoda, A., Sakaki, Y., Takeda, S. & Yoshiura, Y. (2011). Myostatin-deficient medaka exhibit a double-muscling phenotype

with hyperplasia and hypertrophy, which occur sequentially during post-hatch development. *Developmental biology,* 359**:** 82-94.

Cho, S.W., Kim, S., Kim, Y., Kweon, J., Kim, H.S., Bae, S. & Kim, J.-S. (2014). Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome research,* 24**:** 132-141.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W. & Marraffini, L.A. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science,* 339**:** 819-823.

Cradick, T.J., Fine, E.J., Antico, C.J. & Bao, G. (2013). CRISPR/Cas9 systems targeting β-globin and CCR5 genes have substantial off-target activity. *Nucleic acids research***:** gkt714.

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

Dunham, R.A. & Masser, M.P. (2012). *Production of hybrid catfish*: Southern Regional Aquaculture Center.

Edvardsen, R.B., Leininger, S., Kleppe, L., Skaftnesmo, K.O. & Wargelius, A. (2014). Targeted mutagenesis in Atlantic salmon (*Salmo salar* L.) using the CRISPR/Cas9 system induces complete knockout individuals in the F0 generation. *PloS one,* 9**:** e108622.

Erickson, P.A., Ellis, N.A. & Miller, C.T. (2016). Microinjection for Transgenesis and Genome Editing in Threespine Sticklebacks. *JoVE (Journal of Visualized Experiments)***:** e54055-e54055.

Feng, C., Nita-Lazar, M., González-Montalbán, N., Wang, J., Mancini, J., Ravindran, C., Ahmed, H. & Vasta, G.R. (2015). Manipulating galectin expression in zebrafish (Danio rerio). *Galectins: Methods and Protocols***:** 327-341.

Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K. & Sander, J.D. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature biotechnology,* 31**:** 822-826.

Gawecka, J.E., Marh, J., Ortega, M., Yamauchi, Y., Ward, M.A. & Ward, W.S. (2013). Mouse zygotes respond to severe sperm DNA damage by delaying paternal DNA replication and embryonic development. *PLoS One,* 8**:** e56385.

Hanson, T. & Sites, M.D. (2015). 2014 US Catfish Database. *Fisheries and Allied Aquacultures Department Series No 1.* Retrieved from http://www.agecon.msstate.edu/whatwedo/budgets/docs/catfish2014.pdf

Hisano, Y., Sakuma, T., Nakade, S., Ohga, R., Ota, S., Okamoto, H., Yamamoto, T. & Kawahara, A. (2015). Precise in-frame integration of exogenous DNA mediated by CRISPR/Cas9 system in zebrafish. *Scientific reports,* 5.

Ho, C.S., Grange, R.W. & Joho, R.H. (1997). Pleiotropic effects of a disrupted K+ channel gene: reduced body weight, impaired motor skill and muscle contraction, but no seizures. *Proceedings of the National Academy of Sciences,* 94**:** 1533-1538.

Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X. & Shalem, O. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature biotechnology,* 31**:** 827-832.

Iliakis, G., Blöcher, D., Metzger, L. & Pantelias, G. (1991). Comparison of DNA double-strand break rejoining as measured by pulsed field gel electrophoresis, neutral sucrose gradient centrifugation and non-unwinding filter elution in irradiated plateau-phase CHO cells. *International journal of radiation biology,* 59**:** 927-939.

Irion, U., Krauss, J. & Nüsslein-Volhard, C. (2014). Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. *Development,* 141**:** 4827-4830.

Jackson, D.C. (2004). 2 Natural history and fisheries. *Developments in Aquaculture and Fisheries Science,* 34**:** 15-35.

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

Kim, D., Bae, S., Park, J., Kim, E., Kim, S., Yu, H.R., Hwang, J., Kim, J.-I. & Kim, J.-S. (2015). Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nature methods,* 12**:** 237-243.

Kimura, Y., Hisano, Y., Kawahara, A. & Higashijima, S.-I. (2014). Efficient generation of knock-in transgenic zebrafish carrying reporter/driver genes by CRISPR/Cas9-mediated genome engineering. *Scientific reports,* 4.

Lee, C.M., Cradick, T.J., Fine, E.J. & Bao, G. (2016). Nuclease Target Site Selection for Maximizing On-target Activity and Minimizing Off-target Effects in Genome Editing. *Molecular Therapy,* 24**:** 475-487.

Liu, S., Li, Y., Qin, Z., Geng, X., Bao, L., Kaltenboeck, L., Kucuktas, H., Dunham, R. & Liu, Z. (2016a). High-density interspecific genetic linkage mapping provides insights into genomic incompatibility between channel catfish and blue catfish. *Animal genetics,* 47**:** 81-90.

Liu, Z., Liu, S., Yao, J., Bao, L., Zhang, J., Li, Y., Jiang, C., Sun, L., Wang, R. & Zhang, Y. (2016b). The channel catfish genome sequence provides insights into the evolution of scale formation in teleosts. *Nature communications,* 7.

Lliakis, G. (1991). The role of DNA double strand breaks in Ionizing radiation-induced killing of eukaryotic cells. *Bioessays,* 13**:** 641-648.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., Dicarlo, J.E., Norville, J.E. & Church, G.M. (2013). RNA-guided human genome engineering via Cas9. *Science,* 339**:** 823-826.

Mohandas, P., Budell, W.C., Mueller, E., Au, A., Bythrow, G.V. & Quadri, L.E. (2016). Pleiotropic consequences of gene knockouts in the phthiocerol dimycocerosate and phenolic glycolipid biosynthetic gene cluster of the opportunistic human pathogen *Mycobacterium marinum*. *FEMS microbiology letters,* 363**:** fnw016.

Moreno-Mateos, M.A., Vejnar, C.E., Beaudoin, J.-D., Fernandez, J.P., Mis, E.K., Khokha, M.K. & Giraldez, A.J. (2015). CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. *Nature methods,* 12**:** 982-988.

Nunez, M., Villalobos, M., Olea, N., Valenzuela, M., Pedraza, V., Mcmillan, T. & De Almodovar, J.R. (1995). Radiation-induced DNA double-strand break rejoining in human tumour cells. *British journal of cancer,* 71**:** 311.

Pattanayak, V., Lin, S., Guilinger, J.P., Ma, E., Doudna, J.A. & Liu, D.R. (2013). High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nature biotechnology,* 31**:** 839-843.

Peatman, E., Li, C., Peterson, B.C., Straus, D.L., Farmer, B.D. & Beck, B.H. (2013). Basal polarization of the mucosal compartment in *Flavobacterium columnare* susceptible and resistant channel catfish (*Ictalurus punctatus*). *Molecular immunology,* 56**:** 317-327.

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

Ran, F.A., Hsu, P.D., Lin, C.-Y., Gootenberg, J.S., Konermann, S., Trevino, A.E., Scott, D.A., Inoue, A., Matoba, S. & Zhang, Y. (2013). Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell,* 154**:** 1380-1389.

Rosen, J.N., Sweeney, M.F. & Mably, J.D. (2009). Microinjection of zebrafish embryos to analyze gene function. *JoVE (Journal of Visualized Experiments)***:** e1115-e1115.

Schubert, S., Keddig, N., Hanel, R. & Kammann, U. (2014). Microinjection into zebrafish embryos (*Danio rerio*)-a useful tool in aquatic toxicity testing? *Environmental Sciences Europe,* 26**:** 1.

Shah, A.N., Davey, C.F., Whitebirch, A.C., Miller, A.C. & Moens, C.B. (2015). Rapid reverse genetic screening using CRISPR in zebrafish. *Nature methods,* 12**:** 535-540.

Smith, C., Gore, A., Yan, W., Abalde-Atristain, L., Li, Z., He, C., Wang, Y., Brodsky, R.A., Zhang, K. & Cheng, L. (2014). Whole-genome sequencing analysis reveals high specificity of CRISPR/Cas9 and TALEN-based genome editing in human iPSCs. *Cell stem cell,* 15**:** 12.

Söker, T., Dalke, C., Puk, O., Floss, T., Becker, L., Bolle, I., Favor, J., Hans, W., Hölter, S.M. & Horsch, M. (2008). Pleiotropic effects in Eya3 knockout mice. *BMC developmental biology,* 8**:** 1.

Su, B., Shang, M., Li, C., Perera, D.A., Pinkert, C.A., Irwin, M.H., Peatman, E., Grewe, P., Patil, J.G. & Dunham, R.A. (2015). Effects of transgenic sterilization constructs and their repressor compounds on hatch, developmental rate and early survival of electroporated channel catfish embryos and fry. *Transgenic research,* 24**:** 333-352.

Thongda, W., Li, C., Luo, Y., Beck, B.H. & Peatman, E. (2014). L-rhamnose-binding lectins (RBLs) in channel catfish, *Ictalurus punctatus*: Characterization and expression profiling in mucosal tissues. *Developmental & Comparative Immunology,* 44**:** 320-331.

Varshney, G.K., Pei, W., Lafave, M.C., Idol, J., Xu, L., Gallardo, V., Carrington, B., Bishop, K., Jones, M. & Li, M. (2015). High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. *Genome research,* 25**:** 1030-1042.

Veres, A., Gosis, B.S., Ding, Q., Collins, R., Ragavendran, A., Brand, H., Erdin, S., Cowan, C.A., Talkowski, M.E. & Musunuru, K. (2014). Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing. *Cell stem cell,* 15**:** 27-30.

Wang, W., Liu, X., Gelinas, D., Ciruna, B. & Sun, Y. (2007). A fully automated robotic system for microinjection of zebrafish embryos. *PloS one,* 2**:** e862.

Wu, X., Scott, D.A., Kriz, A.J., Chiu, A.C., Hsu, P.D., Dadon, D.B., Cheng, A.W., Trevino, A.E., Konermann, S. & Chen, S. (2014). Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. *Nature biotechnology,* 32**:** 670-676.

Xiao, A. & Zhang, B. (2016). Generation of Targeted Genomic Deletions Through CRISPR/Cas System in Zebrafish. *Methods in molecular biology (Clifton, NJ),* 1451**:** 65.

Xu, Q. (1999). Microinjection into zebrafish embryos. *Molecular Methods in Developmental Biology: Xenopus and Zebrafish***:** 125-132.

Yin, L., Jao, L.-E. & Chen, W. (2015). Generation of targeted mutations in zebrafish using the CRISPR/Cas system. *VEGF Signaling: Methods and Protocols***:** 205-217.

Yuan, S. & Sun, Z. (2009). Microinjection of mRNA and morpholino antisense oligonucleotides in zebrafish embryos. *JoVE (Journal of Visualized Experiments)***:** e1113-e1113.

Zhou, Y., Zhu, S., Cai, C., Yuan, P., Li, C., Huang, Y. & Wei, W. (2014). High-throughput screening of a CRISPR/Cas9 library for functional genomics in human cells. *Nature,* 509**:** 487-491.

Chapter 4: Effects of cecropin B gene transfer and interspecific hybridization on the resistance to *Ichthyophthirius multifiliis* in channel catfish, *Ictalurus punctatus*, and channel catfish female x blue catfish male, *I. furcatus*, hybrids

Abstract

The resistance of channel catfish (*Ictalurus punctatus*), the channel catfish female X blue catfish (*Ictalurus furcatus*) male (CB) hybrid, and channel catfish and hybrid catfish containing cecropin B gene driven by the cytomegalovirus (CMV) promoter to ich, *Ichthyophthirius multifiliis,* infection was investigated. Two experiments were conducted. Transgenic and non-transgenic channel catfish and CB hybrid catfish fingerlings were used in experiment I while food-size non-transgenic channel catfish and hybrid catfish were used in experiment II. In experiment I, four catfish fingerling groups (total 80 fingerlings) including cecropin transgenic channel catfish, cecropin transgenic CB hybrid catfish, non-transgenic channel catfish and non-transgenic CB hybrid catfish, were challenged communally in the same tank and mortality was recorded in each group. Dead fish were sampled for DNA analysis and necropsied to confirm the cause of death. Transgenic fish were identified by nested PCR amplifying a partial sequence of the CMV promoter then confirmed by DNA sequencing. The fish started to die on the 5th day and cumulative mortality reached 98.7% 12 days after infection. No correlation was detected between body weight and time to death for all groups. Non-transgenic channel catfish lived the shortest time when compared to the three other groups. Survival analysis indicated significant differences between at least two groups ($p < 0.05$). Pairwise comparisons revealed significant

differences between non-transgenic channel catfish and the three other groups ($p < 0.05$). No differences were detected between cecropin transgenic channel catfish, cecropin transgenic CB hybrid catfish and non-transgenic CB hybrid catfish ($p > 0.05$). In experiment II, hybrids had less severe infection than channel catfish ($p < 0.05$). Mortality percentage was 62.4 and 40.2% for channel catfish and hybrid catfish, respectively. Hybrid catfish were more resistant to ich infection and the mean survival time for hybrid catfish was significantly longer (more than five days longer) than channel catfish ($p < 0.001$). The results suggest that genetic enhancement of ich resistance can be accomplished in channel catfish by either cecropin transgenesis or interspecific hybridization. Improving survival time is important as well as survival rate, since extension of survival time provides greater opportunity to apply treatments to stop the ich infection.

1. Introduction

Ich or freshwater white spot disease is a highly contagious parasitic infestation caused by the protozoan *Ichthyophthirius multifiliis*. The disease is a major problem in aquaculture (Hoffman, 1999) resulting in severe economic losses (Traxler et al., 1998), especially for intensive production due to not only mortalities, but also the cost of treatment and control measures. Environmental stressors such as low dissolved oxygen, high density and low water quality can predispose fish to ich (Dickerson, 2006). Infective theronts attack the skin of the fish damaging an important barrier of defense against many invasive pathogens. As a result, the fish becomes more susceptible to infection of many additional fish pathogens such as *Aeromonas hydrophila* (Xu et al., 2012a) and *Edwardsiella ictaluri* (Xu et al., 2012b).

Enhancement of disease resistance would result in higher survival rates and lower losses due to diseases, which should lead to increased productivity, profitability, efficiency and welfare of fish (Dunham, 2009). Disease resistance in aquaculture fish species can be enhanced by selection, crossbreeding, interspecific hybridization and transgenesis (Dunham, 2011).

Genetic variation within and between species may result in different levels of disease resistance in fish (Wiegertjes et al., 1996) which could be exploited to develop disease resistant varieties of fish. Evidence of interspecific and intraspecific variation in resistance to *Ichthyophthirius multifiliis* has been observed in poeciliid and goodeid fishes (Clayton and Price, 1992) and between species of rainbowfish (*Melanotaenia eachamensis* and *Melanotaenia splendida*) (Gleeson et al., 2000). Reciprocal hybrids of *Xiphophorus maculatus* and *Xiphophorus variatus* had significantly lower infection levels for ich when compared to the parental stock (Clayton and Price, 1994).

The hybrid between channel catfish (*Ictalurus punctatus*) females and blue catfish (*Ictalurus furcatus*) males (CB hybrid) is the only hybrid of almost 50 ictalurid hybrids that possesses superior economically important traits compared to both parent species (Smitherman and Dunham, 1985). CB hybrids are superior in growth, resistance to many pathogens, higher survival and production in high density ponds, tolerance to low dissolved oxygen levels, and increased harvestibility and dress out percentage (Dunham and Masser, 2012). Xu et al. (2011) concluded that the resistance to ich was not significantly different among CB hybrids, channel catfish and blue catfish, although the mean observed survival of the CB hybrids was the highest.

Another approach that has been used to enhance disease resistance is the generation of cecropin transgenic fish. Cecropins are antimicrobial peptides that are more effective against

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Gram-negative than Gram-positive bacteria (Moore et al., 1996). Generation of F_1 cecropin B transgenic channel catfish enhanced the resistance to *Flavobacterium columnare* and *Edwardsiella ictaluri* when compared to non-transgenic controls (Dunham et al., 2002). Resistance to *Pseudomonas fluorescens* and *Vibrio anguillarum* was enhanced by the transfer of cecropin gene to the medaka (*Oryzias latipes*) (Sarmasik et al., 2002).

In addition to the antibacterial properties of cecropin, antiviral, antifungal and antiprotozoal activities have been reported. Homozygous cecropin P1 and CF-17 transgenic rainbow trout (*Oncorhynchus mykiss*) exhibited improved resistance to *Aeromonas salmonicida* and infectious hematopoietic necrosis virus (IHNV) (Chiou et al., 2014). Cecropins inhibited the replication of human immunodeficiency virus 1 (HIV-1) by suppressing viral gene expression (Wachinger et al., 1998). *In vitro* antifungal activity of cecropin has been demonstrated against different fungal species including *Aspergillus, Fusarium* (De Lucca et al., 1998, De Lucca et al., 2000) and *Candida* species (Park et al., 1997).

Cecropin also exhibited antiprotozoal activity against *Plasmodium* and *Leishmania* species by reducing or inhibiting cyst development (Boman et al., 1989, Gwadz et al., 1989, Akuffo et al., 1998). *Plasmodium berghei* oocyst production was reduced by 60% in cecropin A transgenic mosquito *Anopheles gambiae* when compared to non-transgenic mosquitos (Kim et al., 2004). There is no information available on whether the resistance to ich could be enhanced by the generation of cecropin B transgenic fish or catfish. The objectives of the current study were to compare cecropin transgenesis, interspecific hybridization and their combination for genetically enhancing the resistance to ich for catfish.

2. Methods

This study was conducted to assess the resistance to ich in different cecropin transgenic and non-transgenic catfish genotypes at the fingerling and food size stages, and was organized into two separate experiments. Experiment I was conducted with fish at the fingerling stage representing 4 genotypes: cecropin transgenic channel catfish, cecropin transgenic CB hybrid catfish, non-transgenic channel catfish and non-transgenic CB hybrid catfish. Experiment II was conducted with non-transgenic channel catfish and non-transgenic CB hybrid catfish at the food size stage. All experimental protocols used in this experiment were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC) before the experiment was initiated.

2.1. Experiment I: Effects of cecropin B gene transfer on the resistance to ich in channel catfish and CB hybrid catfish at the fingerlings stage

Eighty 10-month-old fish comprising 4 different genetic groups, cecropin transgenic channel catfish, cecropin transgenic CB hybrid catfish, non-transgenic channel catfish and non-transgenic CB hybrid catfish, were randomly chosen for this experiment. Four fish were lost during the experiment due to predation or other unknown causes, thus the data was collected on 76 fish. Mean body weight ranged from 7.65 to 10.96 g (Table 11). Cecropin transgenic channel catfish were F_3 cecropin transgenic channel catfish whose F_1 ancestors exhibited improved bacterial disease resistance against *Edwardsiella ictaluri* and *Flavobacterium columnare* (Dunham et al., 2002). Cecropin transgenic hybrid catfish were produced by crossing F_2 cecropin transgenic channel catfish females with a non-transgenic mix strain of blue catfish males.

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Channel catfish came from ten spawns (seven cecropin transgenic, three non-transgenic) while CB hybrids were produced from five spawns (two cecropin transgenic and three non-transgenic). The cecropin gene was driven by the cytomegalovirus (CMV) promoter in all cecropin transgenic catfish. All the cecropin transgenic channel catfish and non-transgenic channel catfish parents used for production of the fish used in the study were from the same line and reared in the same earthen pond. Fingerling fish used in this experiment were reared in aquaria at the Fish Genetics Research Unit, E. W. Shell Fisheries Research Center, Auburn University, AL.

The disease challenge was a blind experiment. The fish were randomly chosen for the challenge without identifying the genotype which resulted in unequal numbers of fish in the four groups. After the challenge was completed and time of death was recorded for each fish, tissue samples were analyzed to identify the classification of each fish. Fish were moved to a 200L challenge tank in a flow through system one week before the challenge was initiated. They were kept communally in the same tank with continuous water flow. All fish were healthy and free of any disease symptoms before the experiment was initiated.

A cohabitation challenge was conducted by placing eight ich-moribund catfish obtained from a natural outbreak into the challenge tank. The fish used for infection were excluded from the analysis. Dissolved oxygen and temperature were measured daily. During the entire experiment, dissolved oxygen was above 5 ppm using air supplied from air blowers. Temperature ranged from 19 $^\circ$C at the beginning of the experiment and reached 26 $^\circ$C at the end of experiment. Dead fish were collected twice per day, morphologically identified as channel catfish or CB hybrid catfish, weighed and the anal fin sampled for DNA analysis. Dead fish were necropsied to confirm the cause of death. No other disease infections were observed during the

challenge and no treatment was applied for ich in this experiment. This experiment was terminated at day 20. All the fish died except one cecropin transgenic channel catfish.

Table 11 Number of fish, mean body weight (g) ± standard deviation (SD), estimated mean survival time to death ± standard error (SE) and 95% confidence intervals for cecropin transgenic channel catfish (*Ictalurus punctatus*), non-transgenic channel catfish, cecropin transgenic channel catfish female X blue catfish (*Ictalurus furcatus*) male (CB) hybrid catfish and non-transgenic CB hybrid catfish challenged with *Ichthyophthirius multifiliis*. Estimated mean survival time for non-transgenic channel catfish was significantly different ($p < 0.05$) from the other three groups in which no differences were detected ($p > 0.05$).

Group	Number of fish	Mean body weight (g) ± SD	Mean time to death (days)			
			Estimate	SE	95% confidence interval	
Cecropin transgenic channel catfish	23	10.96 ± 3.49	8.7[a]	0.6	7.5	9.8
Non-transgenic channel catfish	13	7.71 ± 2.79	7.1[b]	0.4	6.4	7.9
Cecropin transgenic CB hybrid catfish	25	7.65 ± 2.98	8.3[a]	0.4	7.6	8.9
Non-transgenic CB hybrid catfish	15	7.57 ± 2.44	8.7[a]	0.4	8.0	9.5

[ab]Means in the same column with same letter are not significantly different (Log rank test, $p > 0.05$) while means with different letters are significantly different (Log rank test, $p < 0.05$).

2.2. Experiment II: Evaluation of interspecific hybridization as an approach to improve the resistance to ich infections in food fish stage channel catfish and CB hybrid catfish

This experiment was conducted with non-transgenic channel catfish and CB hybrid catfish from the same family and genotype as those in experiment I, but the fish were at the food size stage. The channel catfish and CB hybrid catfish used in this experiment were half-sibs (having the same channel catfish female parent). Fish used in experiment II were reared in a 400 m^2 earthen pond for two years. At the time of stocking, all fry had the same age and approximately the same size (~80 mg). After the 2-year rearing period, fish were seined and moved to a holding facility at the Fish Genetics Research Unit, E. W. Shell Fisheries Research Center, Auburn University, AL. Fish were randomly split into three rectangular tanks (837 liters each) so that each tank had random numbers of both channel catfish and CB hybrid catfish. The number of fish from each genotype in each tank can be found in Table 12.

Table 12 Number and mean body weight (kg) ± standard deviation (SD) of channel catfish (*Ictalurus punctatus*) and channel catfish female X blue catfish (*Ictalurus furcatus*) male hybrid (CB hybrid) catfish used in experiment II. Fish in tanks 1 and 2 had a natural outbreak of *Ichthyophthirius multifiliis* while tank 3, the control, did not.

Location	Channel catfish		Hybrid catfish	
	Number	Mean body weight ± SD	Number	Mean body weight ± SD
Tank 1	78	0.544 ± 0.20	51	0.717 ± 0.24
Tank 2	47	0.770 ± 0.30	46	0.841 ± 0.23
Tank 3 (control)	49	0.576 ± 0.27	56	0.753 ± 0.26

While in the holding tank, the fish had continuous water flow from the same source, were fed to satiation with regular catfish floating feed (32% protein), and the dissolved oxygen levels were kept above 5 ppm during the whole experiment using air supplied from air blowers. In March 2016, an outbreak of ich infection occurred in two of the three tanks at around the same time. Before the ich outbreak, the fish were healthy and did not show any signs of disease. Fish in the third tank (control) did not show any disease symptoms and no mortality was recorded in this tank during the entire experiment.

The number of parasite trophonts in the sick fish skin was visually estimated to assess the severity or the level of infection. Since all fish had white spots on their bodies, but with variable numbers, three levels of infection were measured. In level 1, less than 100 trophonts were visible on the fish skin while in level 2, 100 – 300 parasite trophonts were visible on the skin. Level 3 was heavily infected fish whose skin had more than 300 parasite trophonts (Figure 28).

Figure 28 White spots on the skin of sick channel catfish (*Ictalurus punctatus*) and channel catfish female X blue catfish (*Ictalurus furcatus*) male hybrid (CB hybrid) catfish caused by the infection with *Ichthyophthirius multifiliis* at food size. Blue arrow points to a channel catfish that is heavily infected with ich trophonts.

When mortality began, the fish in the two tanks containing infected fish had static treatment with 50 ppm formalin bath for 1 hour /day. After the 1-hour formalin bath, water flow was resumed. Formalin treatment was applied until the mortality stopped and disease symptoms disappeared. Dead fish were collected twice/day, weighed, identified for species and sex, sampled for DNA analysis to confirm the genetic group, necropsied to confirm the cause of death and assigned a value representing the day of death. Since this was a natural outbreak, it was difficult to determine the exact time in which the fish were first exposed to the infection. To overcome this problem, the day in which the white spots were discovered on the fish was

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assumed to be day 0. Experiment II was terminated at day 35 when the fish recovered completely and no fish died in the two tanks for nine successive days.

2.3. Identification of parent species, hybrids and cecropin transgenic fish

2.3.1.   Sample collection and DNA extraction:

Anal fin clips (10-20 mg) were placed into sterile 1.5 ml Eppendorf tubes, individually labelled and then stored at -80 $^\circ$C until analysis. DNA was extracted as follows: Tissue samples were digested in 600 µl of cell lysis buffer (100 mM NaCl, 10 mM Tris, 25mM EDTA, 0.5% SDS and 0.1 mg/ml proteinase K) in a shaker incubator for 60-90 min. Protein was precipitated by adding 200 µl of protein precipitation solution (Qiagen, Redwood City, CA). DNA was then precipitated with isopropanol, washed with 75% ethanol and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA integrity was checked with 1% agarose gel electrophoresis. DNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE).

2.3.2.   Identification of channel catfish and hybrid catfish:

In addition to the identification of channel catfish and hybrid catfish by external appearance, they were also identified anatomically and molecularly. The shape of the swim bladder was examined in dead fish. Channel catfish had a heart shaped, single-lobed swim bladder, while hybrid catfish had a bi-lobed swim bladder with the anterior lobe being heart shaped with the posterior lobe being a small protrusion (Dunham et al., 1982, Dunham and Masser, 2012). Molecular identification of channel catfish and hybrid catfish was achieved by amplifying follistatin (Fst) and hepcidin antimicrobial (Hamp) genes using specific primers (Table 13) (Waldbieser and Bosworth, 2008). Hybrid catfish were heterozygous (Figure 29).
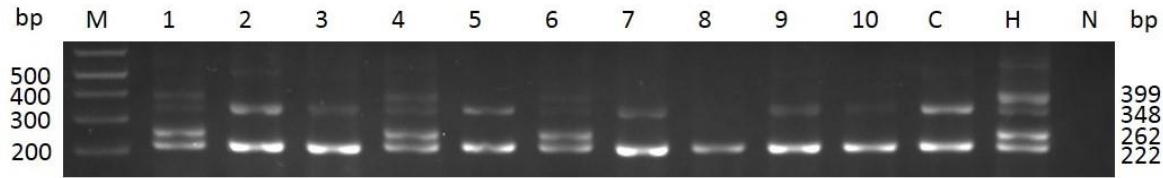
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Figure 29 Amplification of follistatin (Fst) and hepcidin antimicrobial (Hamp) genes from channel catfish (*Ictalurus punctatus*) and channel catfish female X blue catfish (*Ictalurus furcatus*) male (CB) hybrid catfish. Lane M contains 1 kb plus DNA ladder (Invitrogen). Channel catfish had only one band for each of Fst and Hamp, 348 and 222 bp respectively. CB hybrid catfish had two bands for each gene, 399 and 348 bp for Fst and 262 and 222 bp for Hamp. Lanes 1-10 are catfish samples. Lanes C and H are the channel catfish and hybrid catfish controls, respectively. N is a no-template control.

2.3.3. Identification of transgenic catfish:

Nested Polymerase Chain Reaction (PCR):

To ensure PCR specificity, the fish were analyzed for the presence or absence of the CMV-cecropin transgene in a two-step nested PCR targeting CMV promoter. Primers (Table 13) were designed using OligoPerfect™ Designer (Invitrogen). The first-step of the nested PCR was carried out with a 10 µl reaction in a 0.5 mL microcentrifuge tube with the following components: up to 10 µl PCR grade water; 1X PCR buffer without Mg (provided with the enzyme); 2 µM of dATP, dCTP, dGTP and dTTP each (Denville Scientific Inc., Metuchen, NJ); 1.5 mM $MgCl_2$; 0.2 µM CMV 1F; 0.2 µM CMV 1R; 0.2 unit of Platinum® Taq DNA Polymerase (Invitrogen) and 100-300 ng genomic DNA. PCR cycling conditions were as follows: initial denaturation at 94 °C for 4 min; 35 cycles of denaturation at 94 °C for 30 sec,

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annealing at 58 °C for 20 sec, extension at 72 °C for 35 sec; and final extension at 72 °C for 10 min.

In the second-step of nested PCR, 0.5 µl of the first PCR product was used as a template. Primers CMV 2F and CMV 2R were used to amplify a 305 bp region (Fig. 2) that was included in the product amplified in the first-step PCR. A positive control was included by addition of 10 ng of pRC/CMV plasmid DNA construct to the PCR reaction. Negative controls included DNA from non-transgenic channel catfish and non-transgenic hybrid catfish. A contamination control reaction was also included by addition of 0.5 µl of TE buffer instead of the genomic DNA since the genomic DNA from the fish tissue samples was dissolved in TE buffer. To check the PCR result, 1.5% agarose gel electrophoresis was utilized and the samples run for 30 min at 5V/cm. Agarose gels were then visualized with a Molecular Imager® Gel Doc™ XR+ System using Image Lab™ software (Bio-Rad Laboratories, Inc., California, USA). Catfish samples were compared to the positive control as well as the negative controls. Negative controls including non-transgenic channel catfish DNA and non-transgenic CB hybrid catfish DNA were used to confirm the absence of amplification product in negative catfish samples. A no-template control was used to monitor contamination of PCR screening procedures.

Table 13 Primers used to identify fish species and transgenic fish used in the *Ichthyophthirius multifiliis* challenge. Follistatin (Fst) and hepcidin antimicrobial (Hamp) forward (F) and reverse (R) primers were used to identify fish genetic type (channel catfish, *Ictalurus punctatus*, and channel catfish female X blue catfish *Ictalurus furcatus* male (CB) hybrid catfish). Cytomegalovirus (CMV) primers were designed to amplify a partial sequence of CMV promoter in a 2-step nested PCR. CMV 1F (forward) and CMV 1R (reverse) were used in the first step of nested PCR while CMV 2F and CMV 2R were used in the second step. Annealing sites for CMV

2F and CMV 2R primers were present in the PCR product that was amplified by CMV 1F and CMV 1R. Primers CMV 2F and CMV 2R were also used for DNA sequencing to confirm the PCR results.

Primer name	Sequence 5'-3'	Product length (bp)	Reference
Fst F	ATAGATGTAGAGGAGCATTTGAG	348 for channel catfish, 399 for blue catfish	(Waldbieser and Bosworth, 2008)
Fst R	GTAACACTGCTGTACGGTTGAG		
Hamp F	ATACACCGAGGTGGAAAAGG	222 for channel catfish, 262 for blue catfish	
Hamp R	AAACAGAAATGGAGGCTGGAC		
CMV 1F	CCAATAGGGACTTTCCATTGAC	448	This dissertation
CMV 1R	CCAGTTAAGCAGTGGGTTCTCT		
CMV 2F	CCCACTTGGCAGTACATCAA	305	This dissertation
CMV 2R	GGCGGAGTTGTTACGACATT		

DNA sequencing and sequence alignment:

To confirm the presence of the transgene, positive samples were sequenced. A two-step nested PCR reaction was carried out using Expand High Fidelity[PLUS] PCR System (Roche Diagnostics, Indianapolis, IN, USA) with the following components: up to 25 µl PCR grade water; 1X Expand HiFi[PLUS] reaction buffer with $MgCl_2$, 0.2 mM dNTP mix, 0.4 µM of CMV 1F and CMV 1R for the first-step PCR; 0.4 µM of CMV 2F and CMV 2R for the second-step PCR, 100-300 ng genomic DNA for the first reaction, 1 µl of PCR product as a template for the second reaction and 1.25 units of Expand HiFi[PLUS] enzyme blend with the same PCR cycling conditions and the reaction controls. To purify the amplified DNA fragment, samples were run on a 1.5% agarose gel electrophoresis, the 305 bp band was excised into a clean 1.5 mL Eppendorf tube and then purified using QIAquick Gel Extraction Kit (Qiagen). Purified fragments were sequenced from both ends using CMV 2F and CMV 2R primers. To ensure DNA sequencing accuracy and

quality, pRC/CMV was included in two sequencing reactions. Sequencing reactions were performed on an ABI 3100 Genetic Analyzer (Applied Biosystems). Partial CMV promoter sequences obtained from sequencing reactions were trimmed and aligned to the original CMV sequence in the pRC/CMV cecropin expressing vector using t-coffee (Notredame et al., 2000).

2.4. Statistical analysis

The severity of infection (demonstrated by the number of white spots on the fish skin) for channel catfish and CB hybrid catfish was compared using a Chi square contingency table test with Bonferroni adjustment for pairwise comparisons (GarcIa-pErez and NUNez-antOn, 2003). To calculate the mean survival time to death, dead fish were assigned the actual time (day) of death. In experiment I, the day in which the experiment was initiated was given the value zero. The fish that survived were assigned a value of 20 (the day when the experiment was terminated). In experiment II, the day at which the white spots were observed on the fish was considered day 0. Cumulative % mortality was calculated based on the cumulative number of dead fish every day divided by the total number of fish at the beginning of the experiment for each group multiplied by 100.

Mean survival time to death $\pm$ SE (Table 11) was calculated for each group. First, a Pearson product-moment correlation coefficient was computed to rule out any possible effects of body weight on the survival time to death and assess the relationship between the body weight and time of death for all the fish used in the study, regardless of the group. Then the same coefficient was computed for each group separately to assess the relationship between the fish body weights in each group and the time of death.

In survival analysis, alive fish were censored (the actual survival time is partially known, if the experiment continued, the survival time of alive fish would have changed). To investigate the effects of classification and cecropin on survival time, the Kaplan-Meier estimator for survival time to death was computed (Kaplan and Meier, 1958). Log rank (Mantel-Cox) test was used for pairwise comparisons of the survival curves of different genetic groups. To rule out the possibility of considering the surviving cecropin transgenic channel catfish in experiment I as potential outlier, survival analysis was performed two times, with and without the surviving cecropin transgenic channel catfish in the analysis. All analyses were performed using SPSS software version 16.0.

3. Results

3.1. Nested PCR and DNA Sequencing

Nested PCR successfully amplified a 305 bp region of the CMV promoter as shown in Figure 30. A strong specific band was clearly visible for transgenic individuals, which matched the amplified product of the pRC/CMV positive control plasmid DNA. DNA sequencing of transgenic catfish samples proved the presence of CMV in the amplified PCR product. The sequencing chromoatogram showed evenly-spaced single peaks of one color with little baseline noise (Figure 31). Alignment of DNA sequences of transgenic catfish samples revealed 99 % - 100% identity with the CMV sequence.
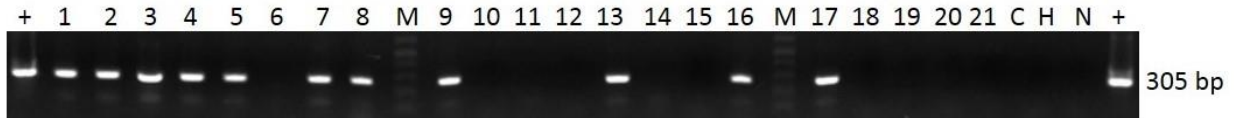
Figure 30 Results of nested PCR amplification of a partial segment of cytomegalovirus (CMV) promoter demonstrated by the 305 bp band in the positive control (+). Lanes M represents 1 Kb plus DNA ladder (Invitrogen). Catfish amplification products are in lanes 1 through 21 where cecropin transgenic channel catfish (*Ictalurus punctatus*) and cecropin transgenic channel catfish female X blue catfish (*Ictalurus furcatus*) male (CB) hybrid catfish showed a clear strong 305 bp band while the band was absent for non-transgenic channel catfish and non-transgenic CB hybrid catfish. Controls used in the PCR reaction were pRC/CMV plasmid DNA (+), non-transgenic channel catfish DNA (C), non-transgenic CB hybrid catfish DNA (H) and no-template control (N).
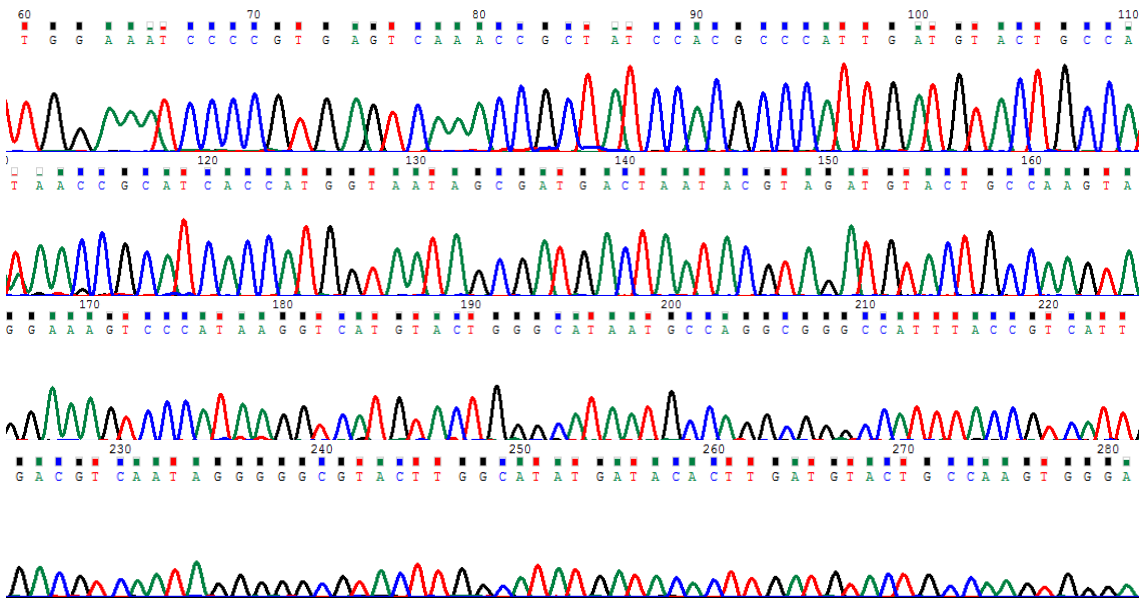

Figure 31 A DNA sequencing chromatogram of a partial sequence of CMV promoter amplified by nested PCR using Expand High Fidelity[PLUS] PCR System (Roche Diagnostics, USA) performed on ABI 3100 Genetic Analyzer (Applied Biosystems) using CMV 2R primer.

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3.2. Experiment I

The cohabitation challenge was effective and resulted in infection. White spots became visible on the fish skin 2-3 days post infection, and catfish started to die the 5th day after the infection. Mortality continued for eight days, reached 98.7%, and then stopped. Only one fish, a cecropin transgenic channel catfish, survived until the experiment was terminated at day 20. Figure 32 demonstrates daily cumulative % mortality for each of the four genetic groups.
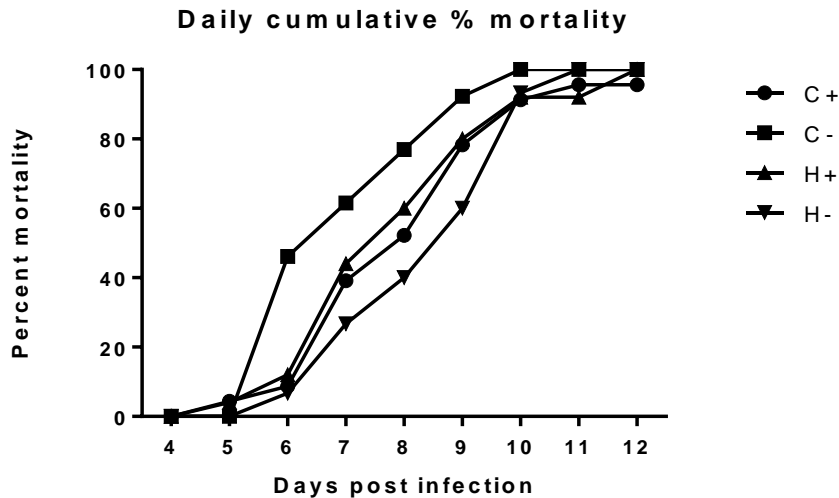

Figure 32 Daily cumulative % mortality from ich infection for 4 catfish groups: cecropin transgenic channel catfish (*Ictalurus punctatus*) (C+), non-transgenic channel catfish (C-), cecropin transgenic channel catfish female X blue catfish (*Ictalurus furcatus*) male (CB) hybrid catfish (H+) and non-transgenic CB hybrid catfish (H-). Daily cumulative % mortality was calculated based on the cumulative number of dead fish everyday divided by the total number of fish at the beginning of the experiment for each group multiplied by 100. Mortality started at the 5th day after infection was induced and continued for eight days. Non-transgenic channel catfish was the first group to reach the 100% daily cumulative mortality followed by non-transgenic CB

hybrids and then cecropin transgenic CB hybrids. One cecropin transgenic channel catfish survived till the experiment was terminated.

No correlation was detected between body weight and time to death for all groups ($r = 0.039$, n = 76, $p = 0.736$), or within cecropin transgenic channel catfish ($r = -0.036$, n = 23, $p = 0.872$), cecropin transgenic hybrid catfish ($r = -0.016$, n = 24, $p = 0.941$), non-transgenic channel catfish ($r = 0.080$, n = 14, $p = 0.786$), and non-transgenic hybrid catfish ($r = -0.034$, n = 15, $p = 0.904$).

Kaplan-Meier plot of survival curves (Figure 33) and pairwise comparisons (Table 11) revealed the difference between non-transgenic channel catfish and the other three genetic groups. Non-transgenic channel catfish lived the shortest time when compared to cecropin transgenic channel catfish ($p = 0.032$), cecropin transgenic CB hybrid catfish ($p = 0.045$) and non-transgenic CB hybrid catfish ($p = 0.009$). No differences were detected between cecropin transgenic channel catfish, cecropin transgenic CB hybrid catfish and non-transgenic CB hybrid catfish ($p > 0.05$, Table 11). Inclusion or exclusion of the surviving cecropin transgenic channel catfish did not alter the outcome of the statistical analysis.
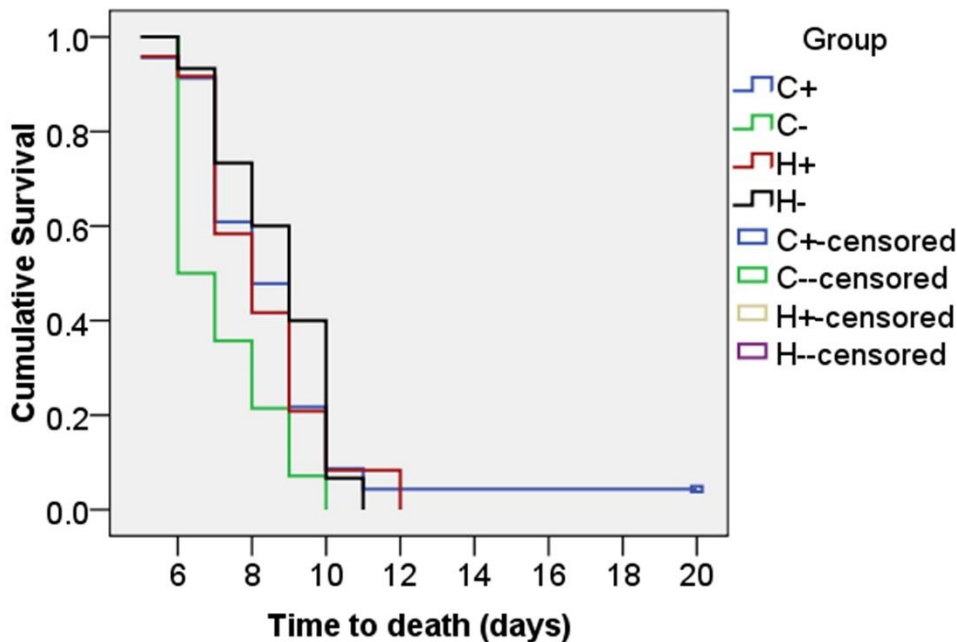
Figure 33 Kaplan-Meier plot of survival curves for dead and alive (censored) catfish in the four groups challenged with *Ichthyophthirius multifiliis*: cecropin transgenic channel catfish (*Ictalurus punctatus*) (C+), non-transgenic channel catfish (C-), cecropin transgenic channel catfish female X blue catfish (*Ictalurus furcatus*) male (CB) hybrid catfish (H+) and non-transgenic CB hybrid catfish (H-). Mortality started at the 5th day after infection was induced and continued for eight days. One cecropin transgenic channel catfish survived till the experiment was terminated (censored at day 20).

3.3. Experiment II

By estimating the approximate number of parasite trophonts on the fish skin, the number and % of fish from each genotype in an infection level was calculated (Table 14, Figure 34). In infection level 1, a higher percent of hybrid catfish (37.1%) existed in this category when compared to channel catfish which had 20% ($p = 0.005$). In level 2 category, no significant differences in the percent of channel and hybrid catfish were detected ($p = 0.317$).
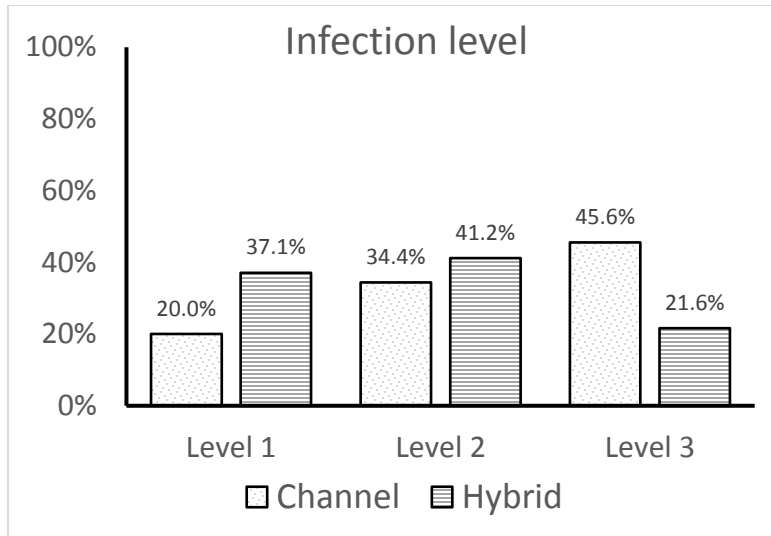
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Figure 34 Level of infection in channel catfish (*Ictalurus punctatus*) and channel catfish female

X blue catfish (*Ictalurus furcatus*) male hybrid (CB hybrid) catfish infected with

Ichthyophthirius multifiliis. Three levels of infection were assumed. In level 1, less than 100

trophonts were visible on the fish skin while in level 2, 100 – 300 parasite trophonts were visible

on the skin. Level 3 was reported in heavily infected fish whose skin had more than 300 parasite

trophonts. Data labels represent the percent of a genotype that had certain infection level out of

the total for that genotype e.g. 20% of the total channel catfish had infection level 1. At the end

of the experiment, no white spots were seen on any survivors.

No correlation was detected between body weight and time to death for channel catfish

and CB hybrid catfish ($r = 0.004$, n = 222, $p = 0.954$), or within channel catfish ($r = -0.116$, n =

125, $p = 0.196$) and CB hybrid catfish ($r = 0.007$, n = 97, $p = 0.947$). Also, no correlation was

detected between the sex of the fish and the time to death ($r = 0.089$, n = 222, $p = 0.188$).

Mortality started five days after the appearance of the white spots on the fish skin (Figure

28) and continued until day 26. Channel catfish had cumulative percent mortality of 62.4%

while CB hybrids had 40.2% mortality (Figure 35). The mean survival time to death for channel

catfish and CB hybrid catfish was significantly different ($p = 0.0003$) (Table 14). CB hybrid catfish were more resistant to ich infection than channel catfish. They lived more than five days longer and had a survival percentage at the end of the experiment that was higher than that of channel catfish by 22.2%. The plot of survival curves for the two genotypes also revealed the difference between channel catfish and CB hybrid catfish (Figure 36).
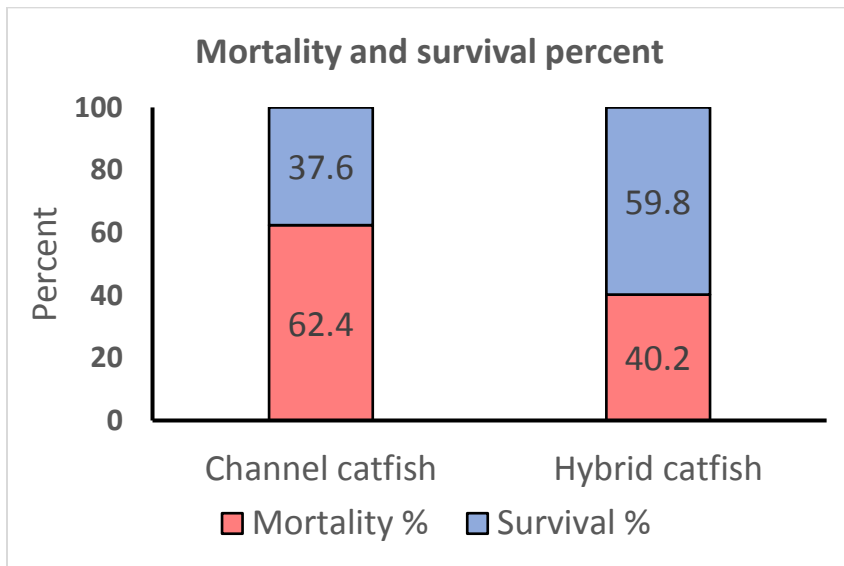
Figure 35 Mortality and survival percent of channel catfish, *Ictalurus punctatus*, and channel catfish female X blue catfish, *Ictalurus furcatus*, male hybrid (CB hybrid) catfish after the infection with *Ichthyophthirius multifiliis*. Mortality percent was calculated as the number of dead fish for a genotype divided by the total number of fish in that genotype at the beginning of the experiment and multiplied by 100. Survival % was calculated as the number of alive fish at the end of the experiment for a genotype divided by the total number of fish at the beginning of the experiment for that genotype and multiplied by 100.
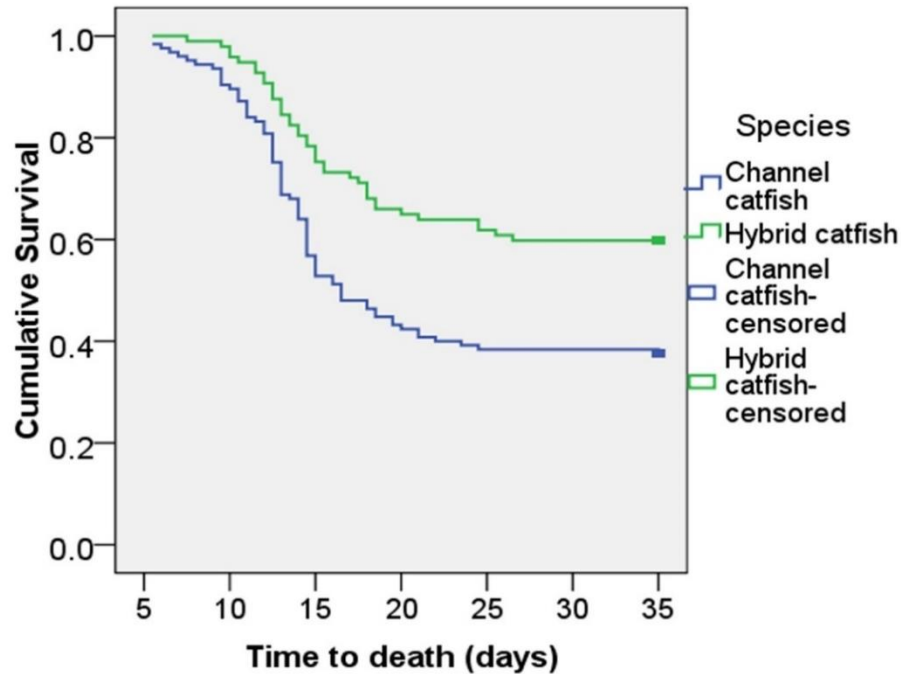
Figure 36 Kaplan-Meier plot of survival curves for dead and alive (censored) non-transgenic

channel catfish, *Ictalurus punctatus*, and channel catfish female X blue catfish, *Ictalurus*

furcatus, male hybrid (CB hybrid) catfish after the infection with *Ichthyophthirius multifiliis*.

Mortality started at day 5 after the appearance of the white spots on the fish body and continued

until day 26. The experiment was terminated at day 35 with alive fish at that time being censored

(the actual survival time is partially known, if the experiment continued, the survival time of

alive fish would have changed).

Table 14 Number, mortality, percent of fish in each infection level and mean survival time to death ± standard error (SE) for channel catfish, *Ictalurus punctatus*, and channel catfish female X blue catfish, *Ictalurus furcatus*, male hybrid (CB hybrid) catfish infected with *Ichthyophthirius multifiliis*. Three levels of infection were measured. In level 1, less than 100 trophonts were visible on the fish skin while in level 2, 100 – 300 parasite trophonts were visible on the skin. Level 3 was reported in heavily infected fish whose skin had more than 300 parasite trophonts. At the end of the experiment, no white spots were seen on all survivors.

Species	Number	Mortality (%)	Fish in each infection level (%)			Survival time to death (days)		
			Level 1	Level 2	Level 3	Mean ± SE	95% confidence interval	
Channel catfish	125	62.4	20.0	34.4[a]	45.6	21.8 ± 0.98	19.9	23.7
Hybrid catfish	97	40.2	37.1	41.2[a]	21.6	27.1 ± 1.02	25.1	29.1

4. Discussion:

Enhancement of disease resistance in farmed fish has been attempted using traditional breeding and transgenic methods. In this study, the possibility of enhancing the resistance to ich infections in catfish using interspecific hybridization of channel catfish female X blue catfish male and transfer of cecropin B gene was evaluated. A natural cohabitation infection showed that hybridization or transfer of the cecropin gene improved ich resistance measured as a length of time until death as fingerlings, but combining the two genetic enhancement programs provided no additional benefit. Hybridization also increased survival at the food size stage during an ich epizootic.

This contrasts with the result for other traits when two successful genetic enhancement programs were combined. Salmon metallothionein (MT) promoter/salmon growth hormone (GH) complementary DNA was transferred to a wild rainbow trout strain (Devlin et al., 2001) and when this GH transgenic wild rainbow trout was crossed with a domestic rainbow trout, the resulting GH transgenic crossbreed was 18 times larger than the non-transgenic wild parent, 13 times larger than the non-transgenic wild X domestic crossbreed, nine times larger than the non-transgenic domestic parent and more than 2.5 times larger than the wild transgenic rainbow trout. The combined effects of transgenesis and crossbreeding had a much greater growth enhancement than crossbreeding or transgenesis alone. Such examples have not been reported for genetic improvement of disease resistance.

In both experiments, hybrid catfish had better resistance to ich infection demonstrated by the survival time to death or the percent of the hybrid catfish that survived at the end of the experiment or both, which is similar to the interspecific hybridization of *Xiphophorus* spp. Clayton and Price (1994) found that reciprocal hybrids of *Xiphophorus maculatus* and *Xiphophorus variatus* had significant lower infection levels for ich when compared to the parental stock.

The cohabitation challenge has been used for ich immunization and ich disease challenges (Dalgaard et al., 2002, Xu et al., 2011). Here, cohabitation challenge was effective in inducing the infection. In both experiments, the fish genotypes under evaluation were placed in the same tank to control variation in the number of infective pathogens, water quality, temperature and feeding rate (Xu et al., 2011). This method is also similar to what happens in fish tanks or ponds where fish are exposed to infective theronts directly from the water.

In experiment I, mortality approached 100% which is similar to previous published cohabitation challenges for ich when channel catfish and CB hybrid catfish were challenged by adding 3 ich moribund fish to the challenge tank (Xu et al., 2011). In experiment II, the infection level ranged from 1 to 3 scale with 1 representing a low level and 3 representing a high level of infection. All fish were infected and white spots were visible on the skin which is consistent with results obtained by Xu et al. (2011), where all the fish showed white spots, although the fish used in their experiment were of different age.

In contrast to our results, Xu et al. (2011) concluded that there were no differences in the susceptibility of channel catfish, blue catfish and CB hybrid catfish to ich. However, with increased replication or a different experimental design, they would have likely reached the same conclusion that the CB hybrid catfish had better survival than the channel catfish, since hybrid had an absolute survival 10% higher than channel catfish. The survival of fish over time after the onset of an epizootic is important since those with a longer survival time have a better chance to receive one or more treatments to combat the disease. If the 10% increased survival observed by Xu et al. (2011) for hybrids was statistically significant or an extended survival time allowed treatment resulting in a 10% better survival, the resulting increase in profit would be $4500 /ha at current (2016) catfish fingerling prices.

Cecropins have been found to have a wide range of effects against different pathogens including bacteria, viruses, fungi and protozoa. Cecropins form large pores in the phospholipid bilayer of the cell membrane (Milani et al., 2009), which is quite similar in different organisms. Additionally, cecropin P1 gene transfer to rainbow trout alters native gene expression for a multitude of immune related genes (Lo et al., 2014). Transfer of cecropin transgene enhanced the resistance to bacterial pathogens in F_1 channel catfish (Dunham et al., 2002) as well as medaka

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(Sarmasik et al., 2002). Recently, cecropin P1 and CF-17 (which contains a synthetic cecropin B analog) were found to enhance the resistance to *Aeromonas salmonicida* and infectious hematopoietic necrosis virus (IHNV) in both F_2 and F_3 transgenic rainbow trout (Chiou et al., 2014).

Cecropins also exhibited antifungal activity against *Aspergillus, Fusarium* (De Lucca et al., 2000) and *Candida* species (Park et al., 1997). The antiprotozoal activity of cecropins included the reduction or inhibition of oocyst production of *Plasmodium* and *Leishmania* species (Gwadz et al., 1989, Akuffo et al., 1998, Kim et al., 2004). Current research indicates that cecropin likely has activity against *Ichthyophthirius*, but additional research is needed to determine the mechanism. Genetic enhancement of catfish by either generation of cecropin transgenic channel catfish or production of CB hybrid catfish is another means of combatting ich epizootics. Generation of cecropin transgenics and interspecific hybridization could be mechanisms to increase resistance to ich in other species of fish.

5. References

Akuffo, H., Hultmark, D., Engstöm, A., Frohlich, D. & Kimbrell, D. (1998). Drosophila antibacterial protein, cecropin A, differentially affects non-bacterial organisms such as Leishmania in a manner different from other amphipathic peptides. *International journal of molecular medicine,* 1**:** 77-159.

Boman, H., Wade, D., Boman, I., Wåhlin, B. & Merrifield, R. (1989). Antibacterial and antimalarial properties of peptides that are cecropin-melittin hybrids. *FEBS letters,* 259**:** 103-106.

Chiou, P.P., Chen, M.J., Lin, C.-M., Khoo, J., Larson, J., Holt, R., Leong, J.-A., Thorgarrd, G. & Chen, T.T. (2014). Production of homozygous transgenic rainbow trout with enhanced disease resistance. *Marine Biotechnology,* 16**:** 299-308.

Clayton, G. & Price, D. (1992). Interspecific and intraspecific variation in resistance to ichthyophthiriasis among poeciliid and goodeid fishes. *Journal of Fish Biology,* 40**:** 445-453.

Clayton, G. & Price, D. (1994). Heterosis in resistance to *Ichthyophthirius multifiliis* infections in poeciliid fish. *Journal of fish biology,* 44**:** 59-66.

Dalgaard, M., Buchmann, K. & Li, A. (2002). Immunization of rainbow trout fry with *Ichthyophthirius multifiliis* sonicate: protection of host and immunological changes. *Bulletin-european association of fish pathologists,* 22**:** 288-297.

De Lucca, A., Bland, J., Jacks, T., Grimm, C. & Walsh, T. (1998). Fungicidal and binding properties of the natural peptides cecropin B and dermaseptin. *Medical mycology,* 36**:** 291-298.

De Lucca, A., Bland, J., Vigo, C., Jacks, T., Peter, J. & Walsh, T. (2000). D-cecropin B: proteolytic resistance, lethality for pathogenic fungi and binding properties. *Medical mycology,* 38**:** 301-308.

Devlin, R.H., Biagi, C.A., Yesaki, T.Y., Smailus, D.E. & Byatt, J.C. (2001). Growth of domesticated transgenic fish. *Nature,* 409**:** 781-782.

Dickerson, H.W. (2006). *Ichthyophthirius multifiliis* and *Cryptocaryon irritans* (Phylum Ciliophora). IN Woo, P.T.K. (Ed.) *Fish diseases and disorders Volume 1: protozoan and metazoan infections.* UK: CAB International.

Dunham, R., Smitherman, R., Brooks, M., Benchakan, M. & Chappell, J. (1982). Paternal predominance in reciprocal channel-blue hybrid catfish. *Aquaculture,* 29**:** 389-396.

Dunham, R.A. (2009). Transgenic fish resistant to infectious diseases, their risk and prevention of escape into the environment and future candidate genes for disease transgene manipulation. *Comparative immunology, microbiology and infectious diseases,* 32**:** 139-61. DOI 10.1016/j.cimid.2007.11.006

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

Dunham, R.A. & Masser, M.P. (2012). Production of hybrid catfish: Southern Regional Aquaculture Center.

Dunham, R.A., Warr, G.W., Nichols, A., Duncan, P.L., Argue, B., Middleton, D. & Kucuktas, H. (2002). Enhanced bacterial disease resistance of transgenic channel catfish *Ictalurus punctatus* possessing cecropin genes. *Mar Biotechnol (NY),* 4**:** 338-44. DOI 10.1007/s10126-002-0024-y

Garcia-Perez, M.A. & Nunez-Anton, V. (2003). Cellwise residual analysis in two-way contingency tables. *Educational and psychological measurement,* 63**:** 825-839.

Gleeson, D., Mccalum, H. & Owens, I. (2000). Differences in initial and acquired resistance to *Ichthyophthirius multifiliis* between populations of rainbowfish. *Journal of Fish Biology,* 57**:** 466-475.

Gwadz, R., Kaslow, D., Lee, J.-Y., Maloy, W., Zasloff, M. & Miller, L. (1989). Effects of magainins and cecropins on the sporogonic development of malaria parasites in mosquitoes. *Infection and Immunity,* 57**:** 2628-2633.

Hoffman, G.L. (1999). Parasites of North American freshwater fishes: Cornell University Press.

Kaplan, E.L. & Meier, P. (1958). Nonparametric estimation from incomplete observations. *Journal of the American Statistical Association,* 53**:** 457-481.

Kim, W., Koo, H., Richman, A.M., Seeley, D., Vizioli, J., Klocko, A.D. & O'brochta, D.A. (2004). Ectopic expression of a cecropin transgene in the human malaria vector mosquito *Anopheles gambiae* (Diptera: Culicidae): effects on susceptibility to Plasmodium. *Journal of medical entomology,* 41**:** 447-455.

Lo, J.H., Lin, C.-M., Chen, M.J. & Chen, T.T. (2014). Altered gene expression patterns of innate and adaptive immunity pathways in transgenic rainbow trout harboring Cecropin P1 transgene. *BMC genomics,* 15**:** 1.

Milani, A., Benedusi, M., Aquila, M. & Rispoli, G. (2009). Pore forming properties of cecropin-melittin hybrid peptide in a natural membrane. *Molecules,* 14**:** 5179-5188.

Moore, A.J., Beazley, W.D., Bibby, M.C. & Devine, D.A. (1996). Antimicrobial activity of cecropins. *Journal of Antimicrobial Chemotherapy,* 37**:** 1077-1089.

Notredame, C., Higgins, D.G. & Heringa, J. (2000). T-Coffee: A novel method for fast and accurate multiple sequence alignment. *Journal of molecular biology,* 302**:** 205-217.

Park, S.S., Shin, S.W., Park, D.-S., Oh, H.W., Boo, K.S. & Park, H.-Y. (1997). Protein purification and cDNA cloning of a cecropin-like peptide from the larvae of fall webworm (*Hyphantria cunea*). *Insect biochemistry and molecular biology,* 27**:** 711-720.

Sarmasik, A., Warr, G. & Chen, T.T. (2002). Production of transgenic medaka with increased resistance to bacterial pathogens. *Marine Biotechnology,* 4**:** 310-322.

Smitherman, R. & Dunham, R. (1985). Genetics and breeding. IN Tucker, C.S. (Ed.) *Channel catfish culture.* Amsterdam: Elsevier Scientific Publishing.

Traxler, G., Richard, J. & Mcdonald, T. (1998). *Ichthyophthirius multifiliis* (Ich) epizootics in spawning sockeye salmon in British Columbia, Canada. *Journal of Aquatic Animal Health,* 10**:** 143-151.

Wachinger, M., Kleinschmidt, A., Winder, D., Von Pechmann, N., Ludvigsen, A., Neumann, M., Holle, R., Salmons, B., Erfle, V. & Brack-Werner, R. (1998). Antimicrobial peptides melittin and cecropin inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression. *Journal of General Virology,* 79**:** 731-740.

Waldbieser, G.C. & Bosworth, B.G. (2008). Utilization of a rapid DNA-based assay for molecular verification of channel catfish, blue catfish, F1 hybrids, and backcross offspring at several life stages. *North American Journal of Aquaculture,* 70**:** 388-395.

Wiegertjes, G.F., Stet, R.M., Parmentier, H.K. & Van Muiswinkel, W.B. (1996). Immunogenetics of disease resistance in fish: a comparative approach. *Developmental & Comparative Immunology,* 20**:** 365-381.

Xu, D.-H., Klesius, P.H., Peatman, E. & Liu, Z. (2011). Susceptibility of channel catfish, blue catfish and channel× blue catfish hybrid to *Ichthyophthirius multifiliis*. *Aquaculture,* 311**:** 25-30.

Xu, D.-H., Pridgeon, J.W., Klesius, P.H. & Shoemaker, C.A. (2012a). Parasitism by protozoan *Ichthyophthirius multifiliis* enhanced invasion of *Aeromonas hydrophila* in tissues of channel catfish. *Veterinary parasitology,* 184**:** 101-107.

Xu, D.-H., Shoemaker, C.A., Martins, M.L., Pridgeon, J.W. & Klesius, P.H. (2012b). Enhanced susceptibility of channel catfish to the bacterium *Edwardsiella ictaluri* after parasitism by *Ichthyophthirius multifiliis*. *Veterinary microbiology,* 158**:** 216-219.