

# Generation of eco-friendly channel catfish, *Ictalurus punctatus*, harboring alligator cathelicidin gene with robust disease resistance by harnessing different CRISPR/Cas9-mediated systems

Jinhai Wang<sup>a</sup>, Baofeng Su<sup>a\*</sup>, De Xing<sup>a</sup>, Timothy J. Bruce<sup>a</sup>, Shangjia Li<sup>a</sup>, Logan Bern<sup>a</sup>, Mei Shang<sup>a</sup>, Andrew Johnson<sup>a</sup>, Rhoda Mae C. Simora<sup>ab</sup>, Michael Coogan<sup>a</sup>, Darshika U. Hettiarachchi<sup>a</sup>, Wenwen Wang<sup>a</sup>, Tasnuba Hasin<sup>a</sup>, Jacob Al-Armanazi<sup>a</sup>, Cuiyu Lu<sup>a</sup>, Rex A. Dunham<sup>a</sup>

<sup>a</sup> School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University, Auburn, AL 36849, United States of America

<sup>b</sup> Current address: College of Fisheries and Ocean Sciences, University of the Philippines Visayas Miagao, Iloilo 5023, Philippines

Corresponding author: Baofeng Su, [bzs0014@auburn.edu](mailto:bzs0014@auburn.edu)

## Abstract

The CRISPR/Cas9 platform holds promise for modifying fish traits of interest as a precise and versatile tool for genome manipulation. To reduce introgression of transgene and control reproduction, catfish species have been studied for upscaled disease resistance and intervening of reproduction to lower the potential environmental risks of introgression of escapees' as transgenic animals. Taking advantage of the CRISPR/Cas9-mediated system, we succeeded in integrating the cathelicidin gene from an alligator (*Alligator sinensis*; *As-Cath*) into the target luteinizing hormone (*LH*) locus of channel catfish (*Ictalurus punctatus*) using two delivery systems assisted by double-stranded DNA (dsDNA) and single-stranded oligodeoxynucleotides (ssODNs), respectively. In this study, high knock-in (KI) efficiency (22.38%, 64/286) but low on-target was achieved using the ssODN strategy, whereas adopting a dsDNA as the donor template led to an efficient on-target KI (10.80%, 23/213). On-target KI of *As-Cath* was instrumental in establishing the *LH* knockout (*LH*<sup>-</sup>*As-Cath*<sup>+</sup>) catfish line, which displayed heightened disease resistance and reduced fecundity compared to the wild-type sibling fish. Furthermore, implanting with HCG and LHRHa can restore the fecundity, spawnability and hatchability of the new transgenic fish line. Overall, we replaced the *LH* gene with an alligator cathelicidin transgene and then administered hormone therapy to gain complete reproductive control of disease-resistant transgenic catfish in an environmentally sound manner. This strategy not only effectively improves the consumer-valued traits, but also guards against genetic contamination. This is a

breakthrough in aquaculture genetics to confine fish reproduction and prevent the establishment of transgenic or domestic genotypes in the natural environment.

## Keywords

Genome editing, ssODN, dsDNA, antimicrobial peptide, immune, reproductive confinement, aquaculture

## 1. Introduction

Innovative biotechnologies continuously develop as science advances, benefiting food production, quality as well as animal and human welfare. Since its inception, CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) has served as a prototype in genome engineering, paving the way for new possibilities in transgenesis and breeding. Two mechanisms are involved for DNA repair when double strand breaks are induced by the CRISPR/Cas9 complex: non-homologous end joining (NHEJ) and homology-directed repair (HDR) [1]. Both mechanism-mediated strategies have been employed in aquaculture to improve the consumer-valued qualities targeted within genetic breeding programs. These harness the NHEJ repair pathway to knock out (KO)/disrupt functional genes or knock in (KI) exogenous genes of interest via HDR at the expected locus to improve the target traits.

Numerous CRISPR/Cas9 systems have emerged recently to improve target-editing efficiency for KI via the HDR pathway. Success has been observed in model animals have been shown successes using ssODN-mediated KIs for the targeted insertions of small DNA fragments since single-stranded oligodeoxynucleotides (ssODNs) act as templates for repairing DNA damage [2-4]. Yoshimi et al. [5] have optimized the ssODN-mediated approach to knock-in larger sequences by the combination of CRISPR/Cas9 system with two 80-bp ssODNs in length. In contrast to conventional plasmid donors, the donor vector used in this system does not require homologous arms (HAs), enabling the insertion of a large vector (CAG-GFP, 4.8 kb) into the designated site (*rRosa26*) with a ~10% integration rate in rat zygotes [5]. Later, using the CRISPR/Cas9-ssODNs mediated KI system, a 10.96% KI efficiency in sheep zygotes was determined [6]. Boel et al. [7] first applied this optimized system to a fish

model, zebrafish (*Danio rerio*), and sequencing results revealed that erroneous repair was more likely to occur when ssODNs were used as repair templates. Alternatively, the modified donor plasmid containing two HAs flanked by two single guide (sgRNA)-targeted sequences (double-cut donors) typically results in a site-specific KI with a high integration rate [8,9], and this HA-mediated KI has been adapted to zebrafish and medaka (*Oryzias latipes*) [9,10]. Theoretically, if we directly offer a linear double-stranded DNA (dsDNA) flanked by two HAs derived from 5'- and 3'-ends of the targeted site and ignore the difference in stability between circular DNA and dsDNA donors, the KI efficiency will increase by convention. In addition to the type of donors, a proper concentration of each component of the CRISPR/Cas9 system has a great positive impact on KI by reducing off-target events and embryo lethality. In this regard, we anticipate achieving extremely efficient KI if a reliable delivery system and an optimized dosage of components are chosen in a non-model fish species.

Currently, transgenesis and CRISPR/Cas9-mediated genome editing have revolutionized traditional theories to accelerate the pace of aquaculture breeding programs, and delivered edible commercial products, such as the genetically modified AquAdvantage salmon [11,12], gene-edited tiger puffer fish and red sea bream (<https://doi.org/10.1038/s41587-021-01197-8>, 2022). Although the NHEJ strategy predominates in altering the consumer-focused traits of fish species, including growth, coloration, and reproduction, the HDR-mediated KI is an effective way to improve the omega-3 fatty acid content and disease resistance [13-15]. In comparison to the non-insertion of KO mutations, the integration of foreign genes by harnessing the HDR pathway usually raises concerns about low KI efficiency and introgression, which directly impact the advocacy of this method and the consumer acceptance of gene-inserted fish [16]. As a result, it is imperative to devise a strategy for both improving the desired traits and preventing introgression to alleviate public concerns about gene-inserted animals. Fortunately, numerous genome-editing-based studies have demonstrated that it is possible to render fish reproductively sterile by altering/disrupting key genes involved in reproduction via the NHEJ repair pathway. Thus, potentially reducing negative environmental effects associated with genetically modified fish [17-19]. Luteinizing hormone (*LH*) gene regulates gametogenesis and gestation through binding the receptor [20,21]. LH-deficient female zebrafish are

infertile, whereas the mutant males are fertile, indicating that the *LH* gene facilitates fish oocyte maturation and triggers ovulation [22]. In addition, interruption of the *LH* gene in channel catfish and white-edged rockfish (*Sebastes taczanowskii*) can result in the production of sterile lines [21,23].

Large-scale disease outbreaks are inevitable, and methods of disease control need to be improved. Antimicrobial peptides (AMPs) are polypeptides that serve as substitutes for antibiotics in a variety of species' initial line of defense (innate immunity) against microbial invasions without developing considerable antibiotic resistance [24,25]. AMPs and antimicrobial peptide genes (AMGs) including cecropin, hepcidin, piscidin, epinecidin-1, lysozyme, and lactoferrin have been used for decades to improve disease resistance in a variety of aquatic animals, as feed supplements or transgenes [13,26]. Cathelicidins are a particularly important AMP family, sharing the common cathelin-like domain [27] and exhibiting broad-spectrum antimicrobial and immune-modulating activities [28]. Recent investigations have shown that alligator-derived cathelicidin inhibits fish pathogens both *in vivo* and *in vitro* [29-31]. Therefore, integrating the AMG into the genomic DNA has broad prospects for establishing novel disease-resistant fish lines.

Fish transgenic for AMGs could provide a significant option to address disease problems, however, and additional goal would be to prevent the possibility of breeding of escapees with wild populations. Hypothetically, a reproductive gene such as *LH*, responsible for gametogenesis and gestation could be knocked out at the DNA level with the replacement of a cathelicidin gene, leading to sterile fish with heightened disease resistance. Genome-edited sterilized fish from this approach would have fertility temporarily restored with hormone therapy used for artificial spawning of fish, and it is achievable to produce environmentally-compatible and disease-resistant fish lines. In this study, two CRISPR/Cas9 delivery systems: HA- and ssODN-mediated KI were employed to insert the *As-Cath* gene at the channel catfish (*Ictalurus punctatus*) *LH* locus to develop a reversibly sterile and disease-resistant line. We compared the KI efficiency, hatchability and fry survival from various systems, and then restored the fertility of As-Cath-integrated sterile of P<sub>0</sub> founders through hormone therapy. In addition, the bacterial resistance of P<sub>0</sub> and F<sub>1</sub> individuals from the new fish line was further evaluated.

## 140 **2. Materials and methods**

### 141 *2.1. Ethical approval*

142

143 The care and use of animals followed the applicable guidelines from expert training  
144 courses. Experimental protocols in the current study were approved by the Auburn  
145 University Institutional Animal Care and Use Committee (AU-IACUC). All fish  
146 studies were conducted in compliance with the procedures and standards established  
147 by the Association for Assessment and Accreditation of Laboratory Animal Care  
148 (AAALAC).

149

### 150 *2.2. Target locus for gene insertion*

151

152 As the target integration site, we selected the *LH* gene, which is widely expressed in  
153 the theca cells of the ovary and aids in egg maturation and ovulation during gonadal  
154 development [22]. Based on the published genome of channel catfish [32], the chosen  
155 *LH* site for sgRNA targeting was located in the middle of exon 2 (Fig. 1(A-B)). The  
156 inserted segment was derived from the coding sequence (CDS) of the cathelicidin  
157 gene of *Alligator sinensis* (*As-Cath*, GeneBank accession number XM\_006037211.3)  
158 [29].

159

### 160 *2.3. Design of donor DNA, sgRNA and CRISPR/Cas9 system*

161 Gene-targeted KI can be engineered via HDR using the dsDNAs or ssODNs as donor  
162 templates. In the current study, we employed two CRISPR/Cas9-mediated systems to  
163 conduct targeted KI of the *As-Cath* fragment at the *LH* locus. For the first system, the  
164 CDS of the *As-Cath* gene was cloned into the pUC57\_mini vector at the EcoRV  
165 enzyme digestion site to create the ssODN1\_*As-Cath*\_ssODN2 construct as a plasmid  
166 donor. Two sgRNAs (sgRNA1 and sgRNA2) were co-injected to operate as “scissors”,  
167 cutting the *LH* gene and linearizing the plasmid donor, respectively, and provided two  
168 short ssODNs to ligate the ends of both cut sites, labeled as the 2H2OP system (Fig.  
169 1(A)). ssODN1 consists of 80 bp, of which the upstream 40 bp are derived from  
170 partial exon 2 of *LH* gene and the remaining 40 bp are homologous to pUC57\_mini  
171 backbone. For ssODN2, the upstream 40 bp are from the pUC57\_mini backbone,  
172 while downstream 40 bps come from a portion of exon 2 of the *LH* gene. The dsDNA  
173 donor was created by constructing the *As-Cath* CDS sequence flanked with two

homology arms (HAs) of 300 bp derived from the *LH* gene of channel catfish on either side of the insert DNA, and we tagged the second construct as HA1-As-Cath-HA2. More specifically, 163 bp of HA1 (the left homology arm) are derived from the upstream of exon 2; 136 bp are identical to intron 1, and 1 bp originated from exon 1. HA2 (the right homology arm) contains 21 bps from exon 2's downstream; 85 bps from intron 2 and 194 bps from upstream of exon 3 (**Appendix A**). Here, we used one sgRNA (sgRNA1) to cut the LH site in the channel catfish genomic DNA and provided a linear dsDNA as the donor template, and this system was labeled as dsDNA (**Fig. 1(B)**). For both constructs, the expression of the *As-Cath* gene was driven by the zebrafish ubiquitin (UBI) promoter [33]. The linear dsDNA, circular plasmid and ssODNs were synthesized by Genewiz (South Plainfield, NJ).

The sgRNAs were selected via the CRISPR design online tool (CRISPR Guide RNA Design Tool, Benchling, <https://zlab.bio/guide-design-resources>) that targeted the *LH* gene of channel catfish and the donor plasmid. Candidate sgRNA sequences were compared to the whole genome of channel catfish via the Basic Local Alignment Search Tool to avoid cleavage of off-target sites. In addition, putative off-target sites were excluded using the online tool Cas-OFFinder (<http://www.rgenome.net/cas-offinder/>) [34]. Eventually, sgRNA1 for LH locus and sgRNA2 for donor plasmid were obtained. The Maxiscript T7 kit (Thermo Fisher Scientific, Waltham, MA) was used to generate sgRNAs *in vitro*, according to the instructions. Then purified sgRNAs were prepared using the RNA Clean and Concentrator Kit (Zymo Research, Irvine, CA). The concentration and quality of sgRNAs were detected with Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and 1% agarose gel with 1 × tris-borate-EDTA (TBE) buffer, respectively. The synthetic sgRNAs were diluted to a concentration of ~ 300 ng/μL and then divided into PCR tubes (2 μL/tube), and stored at -80 °C until use. The Cas9 protein powder was purchased from PNA BIO Inc. (Newbury Park, CA), and was diluted with DNase/RNase-free water to 50 ng/μL, keeping at -20 °C until use. Single guide RNAs and universal primer used in this study are listed in **Table 1**. Two different dosages of the donor DNA template and two control groups were set up: 50 ng/μL, 100 ng/μL, sham-injected control (iCT, only the 10% phenol red solution was injected) and non-injected control (nCT, no injection) for each KI system.

#### 208 2.4. Transgenic fish production and rearing

209

210 Mature channel catfish females and males were paired for artificial spawning  
211 according to [Elaswad et al. \[35\]](#) with some modifications. Briefly, we selected  
212 individuals weighing more than 1.5 kilograms for spawning. Female channel catfish  
213 were implanted with 75 µg/kg of luteinizing hormone-releasing hormone analog  
214 (LHRHa) to induce ovulation, then eggs were gently stripped in a 20-cm greased  
215 spawning pan. Mature males were euthanized; testes were collected, rinsed, weighed  
216 and crushed; and sperm were prepared in 0.9 % saline solution (g:v = 1:10). Two  
217 milliliters of sperm solution were added to approximately 300 eggs and gently mixed.  
218 After a one-minute mixing, sufficient pond water was added to the eggs to activate the  
219 sperm, then the sperm/egg mixture was gently swirled for 30 s. More water was added  
220 and the embryos were kept in a single layer in the pan, and the embryos were allowed  
221 to harden for 15 min before microinjection.

222

223 The CRISPR/Cas9 system used for KI microinjections was combined with Cas9  
224 protein, sgRNA and donor template in the ratio of 2:1:1, including one component of  
225 phenol red as an indicator. For the ssODN1\_As-Cath\_ssODN2 construct (2H2OP  
226 system), 8 µL of Cas9 protein (50 ng/µL), 2 µL of sgRNA1/sgRNA2 (300 ng/µL), 2  
227 µL of donor plasmid (50 ng/µL, 100 ng/µL), 2 µL of ssODN1/ssODN2 (50 ng/µL, 100  
228 ng/µL) and 2 µL of phenol red solution were mixed for microinjection (Total 8 + 2 + 2  
229 + 2 + 2 + 2 + 2 = 20 µL). With respect to the HA1\_As-Cath\_HA2 construct (dsDNA  
230 system), 4 µL of Cas9 protein (50 ng/µL), 2 µL of sgRNA1 (300 ng/µL), 2 µL of  
231 donor dsDNA (50 ng/µL, 100 ng/µL), 2 µL of phenol red and 10 µL of DNase-free  
232 water were mixed to bring it up to 20 µL in total. For each mixture of the  
233 CRISPR/Cas9 system, we mixed Cas9 protein and sgRNA first and incubated them on  
234 ice for 10 min, then the donor templates were supplemented. For the iCT group, we  
235 only injected phenol red (diluted with 0.9 % saline). The mixed solution for each  
236 treatment was microinjected into one-cell stage embryos as previously described [\[36\]](#).  
237 Every 6 µL of the mixture was loaded into a 1.0 mm OD borosilicate glass capillary  
238 that was pulled into a needle by a vertical needle puller (David Kopf Instruments,  
239 Tujunga, CA), and injected into 600 embryos. We injected 1,000 embryos dividing  
240 them into 5 random replicates for each treatment, and another 200 embryos with 3  
241 replicates were prepared for each control group, respectively. All these embryos were



from the same parents, and the microinjection was terminated after 90 min post-fertilization.

All injected and control embryos were transferred into 10-L tubs filled with 7-L Holtfreter's solution (59 mmol NaCl, 2.4 mmol NaHCO<sub>3</sub>, 1.67 mmol MgSO<sub>4</sub>, 0.76 mmol CaCl<sub>2</sub>, 0.67 mmol KCl) [37] and 10–12 ppm doxycycline for hatching immediately after microinjection. All tubs were placed in the same flow-through hatching trough and a heater was put upstream of the trough to ensure that the water temperature was 26–28 °C while dissolved oxygen levels were > 5 ppm via continuous aeration with airstones. Holtfreter's solution was replaced twice per day and dead embryos/fry were collected and recorded daily during hatching to analyze hatchability, fry survival rate and genotype. The hatched fry were transferred to a Holtfreter's solution without doxycycline and fed with live *Artemia* nauplii four times per day. After one week of culture in tubs, all fry from each treatment were stocked separately into 60 L aquaria (120 fish/tank) in a recirculating system for growth experiments. Feed pellet size was adjusted according to the size of the fish's mouth as the fish grew. In detail, fry in tanks fed with Purina® AquaMax® powdered feed (50% crude protein, 17% crude fat, 3% crude fiber, and 12% ash) four times per day for two months. Then fingerlings were fed with Aquaxcel WW Fish Starter 4512 (45% crude protein, 12% crude fat, 3% crude fiber, and 1% phosphorus) twice a day for two months. Juvenile fish were fed with WW 4010 Transition feed (40% crude protein, 10% crude fat, 4% crude fiber, and 1% phosphorus) once a day [14]. All fish were fed to satiation.

## 2.5. Integration analysis and mutation detection

After a 4-month culture, all fingerlings (20–40 g) were pit-tagged (Biomark Inc., Boise, Idaho, USA) to distinguish each individual, the fish from different treatments were then mixed together and randomly dispersed into two circular tanks (1,200 L volume filled with ~800 L of water) with the same density (120 fish/tank) for growth comparison monthly. Meanwhile, the pelvic fin clip and barbel were taken from anesthetized fish for DNA extraction and genotypic identification. During this phase, all fish received WW 4010 Transition feed once a day to satiation. Different genotyping strategies were involved for these two constructs:



ssODN1\_As-Cath\_ssODN2, the CDS region of As-Cath was amplified to confirm gene insertion using primers Cath1-F/R (forward and reverse), and the promoter region was amplified via primers Prom1-F/R. As for the junctions, ssODN1 and ssODN2 regions were amplified using primers ssODN1-F/R and ssODN2-F/R to determine whether it was a target-site insertion. With respect to the HA1\_As-Cath\_HA2 construct, the As-Cath and promoter regions were detected using primers Cath2-F/R and Prom2-F/R, respectively. Then the left HA and right HA junctions were amplified via primers HA1-F/R and HA2-F/R. Primers were designed using the online software Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) and listed in Table S1 (Appendix B). PCR was performed in a 10- $\mu$ L system and PCR products were resolved and visualized by running 1.0% agarose gel with 1  $\times$  tris-acetate-EDTA (TAE) buffer, and a bright band of each region with the corresponding length indicated an on-target positive (LH<sup>-</sup>\_As-Cath<sup>+</sup>). Here, if we can determine that some individuals have been inserted with the As-Cath fragment, but we can not detect the junctional regions (HA- or ssODN-region), we then conclude them as potential off-target positives (LH<sup>+</sup>\_As-Cath<sup>+</sup>).

With respect to the LH<sup>+</sup>\_As-Cath<sup>+</sup> fish, we selected 60 individuals to be tested for LH mutations. In this case, PCR was performed in a 20  $\mu$ L-volume system using Expand High Fidelity<sup>PLUS</sup> PCR System (Roche Diagnostics, Indianapolis, IN, USA) according to Elawad et al. [35], and LH-F/R primers were used in both constructs. Then, the surveyor mutation detection assay was performed via Surveyor Mutation Detection Kit (Integrated DNA Technologies, IDT, Coralville, Iowa, USA) according to the detailed instructions [38]. A negative control reaction was included in the assay by using genomic DNA from the nCT group. Surveyor-digested DNA samples were electrophoresed for 1 hour in a 2% agarose gel using 1  $\times$  TBE buffer and compared to wild-type samples.

## 2.6. DNA sequencing

For the integrated As-Cath, promoter and junction sequences, PCR of positive samples was performed in a 50  $\mu$ L-volume of system. Then the PCR products were purified using the QIAquick<sup>R</sup> PCR Product Purification Kit (QIAGEN, Hilden,

Germany) according to the manufacturer's instructions. Before sequencing, all purified DNA samples were quantitated and identified using Nanodrop and by running 1.0% agarose gel. Primers Cath1-F/Cath2-F and Prom1-F/Prom-2F were used for sequencing of As-Cath and promoter regions for HA1\_As-Cath\_HA2 and ssODN1\_As-Cath\_ssODN2 constructs, respectively; primers HA1-F/HA2-F and ssODN1-F/ssODN2-F were used for sequencing of junctional regions for these two constructs, respectively.

317

Regarding LH mutations, we cloned the PCR products of putative mutant individuals using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) before sequencing following the instructions with some modifications. Briefly, PCR was performed on each mutant individual that was previously identified with Surveyor assay using the primers LH-F/R for the next cloning steps. In addition, the DNA of three wild-type individuals from the nCT group was prepared using the same primers and procedures, then combined into one reaction and cloned as a wild-type control for sequencing. After cloning, we transformed the pCR<sup>TM</sup>4-TOPO vector containing the PCR products into One Shot TOP10 Electrocomp<sup>TM</sup> *E. coli* (Invitrogen, Carlsbad, CA) as previously described [35]. Then 15 single colonies were randomly picked up to perform Colony PCR, and LH-F primer was used for the sequencing of LH mutant samples.

329

## 330 2.7. Determination of mosaicism and transgene expression

331

Five 12-month-old on-target positive fish and five sham-injected control fish were randomly chosen and sacrificed. Fourteen tissues, including skin, liver, kidney, spleen, blood, intestine, gill, stomach, fin, barbel, muscle, eye, brain and gonad of each individual were collected in 1.5 mL tubes and immediately transferred into liquid nitrogen for DNA and RNA isolation. PCR and quantitative real-time PCR (qRT-PCR) were conducted to detect the *As-Cath* gene's potential mosaicism and mRNA level. Total RNAs were isolated from various tissues using TRIzol reagent (Thermo Fisher Scientific) and were reverse transcribed to cDNA using iScript<sup>TM</sup> Synthesis Kit (Bio-Rad, Hercules, CA) following the manufacture protocols.

341

qRT-PCR was performed on a C1000 Thermal Cycler using SsoFast<sup>TM</sup> EvaGreen Supermix kit (Bio-Rad, Hercules, CA) according to the instructions. Concentrations

of the cDNA products were diluted to 250 ng/μL, and 1 μL template was used in a 10 μL PCR reaction volume. The mRNA level of 18S rRNA was used as an internal control, and the detailed qRT-PCR procedure was set up according to Coogan et al. [39]. The primers (Cath\_RT-F and Cath\_RT-R) used for qRT-PCR are listed in Table S1 (Appendix B). The CFX Manager Software (version 1.6, Bio-Rad) was used to collect the raw crossing-point (C<sub>t</sub>) values. The expression level of a target gene to the 18S rRNA gene from transgenic fish against non-transgenic sibling fish was converted to fold differences. Each sample was analyzed in triplicate using the formula  $2^{(-\Delta\Delta CT)}$ , which sets the zero expression of the non-transgenic full-siblings to 1× for comparison.

## 2.8. Reproductive evaluation and restoration of parental KI fish

All P<sub>0</sub> fish were stocked into a 0.04-ha earthen pond at Fish Genetics at Auburn University for growth and maturation. At the age of two years, some P<sub>0</sub> individuals are expected to reach sexual maturity [40]. To evaluate the reproduction of two-year-old KI founders, on-target positive (LH<sup>-</sup>As-Cath<sup>+</sup>), off-target positive (LH<sup>+</sup>As-Cath<sup>+</sup>), and wild-type (WT) fish were selected to conduct a three-round mating experiment. Firstly, 3 pairs of WT, 6 pairs of LH<sup>-</sup>As-Cath<sup>+</sup>, and 4 pairs of LH<sup>+</sup>As-Cath<sup>+</sup> mature parents were randomly placed into 13 tanks (60 × 45 × 30 cm<sup>3</sup>) for a two-week natural spawning to evaluate the spawnability of each genotype, and egg masses were collected from the spawnable parents. Then we primed the males with a 50 μg/kg LHRHa implant and 1600 IU/kg human chorionic gonadotropin (HCG) in the unspawned groups with a one-week observation to determine if LH<sup>-</sup>As-Cath<sup>+</sup> females were fertile. After this period, we recruited 6 more pairs of LH<sup>-</sup>As-Cath<sup>+</sup> fish to perform a 3 × 4 factorial design with 3 dosages of a combination of HCG and LHRHa implant (1200 IU/kg HCG + 50 μg/kg LHRHa, 1600 IU/kg HCG + 50 μg/kg LHRHa, 2000 IU/kg HCG + 50 μg/kg LHRHa) and 0.85% NaCl injected control group to assess the effects of hormone therapy. A 30-g egg mass for each genotype with 3 replicates was collected to calculate the fecundity (eggs/kg body weight [BW]). The masses were then transferred into tubs for hatchability and fry survival determination. Fish were fed ad libitum throughout the experiment.

## 2.9. Generation and genotype analysis for F<sub>1</sub> fish

378

379 All the fry were separated into 60 L tanks by different genotypes. After 4 months of  
380 culture, fin clips and barbels were collected for DNA extraction from 60 F<sub>1</sub>  
381 individuals of each genotype except the control groups. The same culture and  
382 genotyping procedures as described above were applied to the F<sub>1</sub> generation.

383

## 384 2.10. Experimental challenge with *Flavobacterium covaie* and *Edwardsiella ictaluri*

385

386 Gene-edited channel catfish were cultured in 60 L aquariums in the greenhouse of the  
387 Fish Genetics Laboratory at Auburn University (approved by AU-IACUC). To  
388 determine the resistance against pathogens, both P<sub>0</sub> and F<sub>1</sub> fish were challenged by *F.*  
389 *covaie* and *E. ictaluri*.

390

391 *F. covaie* challenge. Healthy P<sub>0</sub> fingerlings with body weight  $150.62 \pm 4.24$  g (mean  $\pm$   
392 SEM), including four genotypes (15 fish/genotype): LH<sup>-</sup>As-Cath<sup>+</sup>, LH<sup>+</sup>As-Cath<sup>+</sup>,  
393 negative LH<sup>+</sup>As-Cath<sup>-</sup> (negative fish without As-Cath insertion or LH mutation) and  
394 WT were mixed and acclimated in one hatching trough for five days and then  
395 transferred to a 1,800-L tank in the challenge room for acclimation for another 24 to  
396 48 h prior to bacterial infections. All fish were randomly/equally separated into two  
397 60-L buckets (30 L water). Briefly, a revived *F. covaie* isolate (strain ALG-00-530) on  
398 modified Shieh agar (MSA) was inoculated into multiple cultures of 12 mL of  
399 modified Shieh broth (MSB) in 50-mL sterile flasks and grown in a shaker incubator  
400 at 150 rpm for 12 hours at 28°C. These cultures were then expanded into 200 mL  
401 cultures (5 mL additions) in 500 mL flasks and grown for another 12 h. The optical  
402 density was adjusted to OD<sub>540</sub> = 0.731 and then spread plate dilutions were performed  
403 to determine the final inoculum concentration. One hundred microliters of each  
404 inoculum were serially diluted and spread onto MSA agar plates in duplicate and  
405 incubated at 28 °C for 48 h to quantify the concentration of the inoculum. Two flasks  
406 containing 325 mL of inocula ( $4.55 \times 10^8$  CFU/mL) were immediately added to two  
407 60 L buckets with fish following preparation, respectively. Then the fish were  
408 immersed statically in buckets for 1.5 hours at ~28 °C (immersion dose:  $2.46 \times 10^6$   
409 CFU/mL); afterward, all fish were gently moved back into the 1,800-L tank  
410 containing 1,000-L water and water flow was resumed. Meanwhile, a  
411 mock-challenged tank was used as the control but incorporated another 40 fish in 30 L

of rearing water for 1.5 hours with sterile modified Shieh broth (325 mL) instead of the bacterial culture. With respect to the challenge of F<sub>1</sub> fry (3.15 ± 0.24 g), four families of F<sub>1</sub> fry (45 fish/family): LH<sup>-</sup>As-Cath<sup>+</sup>, LH<sup>+</sup>As-Cath<sup>+</sup>, LH<sup>+</sup>As-Cath<sup>-</sup> and WT were selected, and each family was randomly divided into three replicates with 15 fish per basket. The same challenge procedure and strain of *F. covae* with a dose of 4.75 × 10<sup>8</sup> CFU/mL (immersion dose: 2.57 × 10<sup>6</sup> CFU/mL) were implanted for the F<sub>1</sub> generation.

*E. ictaluri* challenge. Sixty P<sub>0</sub> fish (142.62 ± 3.72 g) including the above four genotypes, were prepared for the *E. ictaluri* challenge. *E. ictaluri* (S97-773) was provided by the USDA-ARS, Aquatic Animal Health Research Unit, Auburn, AL. The detailed procedures of the *E. ictaluri* challenge were performed according to Simora et al. [30] with some modifications. Briefly, 1 mL of frozen glycerol stock of *E. ictaluri* was inoculated into 20-mL brain–heart infusion broth (BHIB; Hardy Diagnostics) at 26°C in a shaker incubator at 180 rpm for 24 hours. And then bacteria were subcultured into 1-L BHIB for another 24 hours at the same condition until the cell density reached ~1 × 10<sup>8</sup> CFU/mL based on the OD<sub>600</sub> value. All 60 P<sub>0</sub> individuals were transferred into one 1,800-L tank for the challenge. Before starting *E. ictaluri* infection, water was lowered to a total of 100 L, then one liter of *E. ictaluri* suspension containing 3.2 × 10<sup>8</sup> CFU/mL cells was added to the tank resulting in a final immersion dose of 3.2 × 10<sup>6</sup> CFU/mL. Fish were immersed statically for 2 hours with aeration > 5 ppm, then water was restored. In addition to infected groups, one control tank containing 30 fish received only BHIB as a mock-challenged group. With respect to the challenge of F<sub>1</sub> fingerlings (54.27 ± 1.49 g), a total of four genotypes containing 60 fish were selected, and the same challenge procedure and strain of *E. ictaluri* with a dose of 2.8 × 10<sup>8</sup> CFU/mL (immersion dose: 2.8 × 10<sup>6</sup> CFU/mL) were implanted for the F<sub>1</sub> generation.

During the first 72 h of the experiment, we checked for mortality every four hours and then three times daily. Challenged fish were continuously monitored for 10 days for external clinical signs of *F. covae*/*E. ictaluri* and confirmation of bacteria colony growth by isolating bacteria from the kidney and liver to determine the cause of death, and dead individuals were recorded over time.

## 2.11. Statistical analysis

Spawnability, hatchability, fecundity, fry survival rate, and growth data were analyzed using one-way ANOVA/Tukey's multiple comparisons test to determine the mean differences among treatments. To compare the KI efficiency of different groups, one-way ANOVA/Tukey's multiple comparisons and odds ratio (OR) (Table S3 in Appendix B) were adopted. The survival curves of challenge experiments from different genotypes were compared by the Kaplan-Meier plots followed by Log-rank (Mantel-Cox) test. All statistical analysis was achieved via GraphPad Prism 9.4.1 (GraphPad Software, LLC). Gene expression between transgenic and non-transgenic fish was analyzed with an unpaired Student's two-sample *t*-test. Statistical significance was set at  $P < 0.05$ , and all data were presented as the mean  $\pm$  standard error (SEM).

## 3. Results

### 3.1. Targeted KI of *As-Cath* gene into the *LH* locus

Both the 2H2OP and dsDNA systems can induce *As-Cath*-integrated catfish lines with high integrated ratios, but the 2H2OP system had significant off-target effects (Fig. 1(CD), Fig. S1-S4 in Appendix B). More specifically, the 2H2OP system containing 50 ng/ $\mu$ L of donors (2H2OP50) showed the highest KI efficiency at 27.61% (37/134), followed by the groups 2H2OP100 (17.76%, 27/152), dsDNA50 (12.21%, 26/213) and dsDNA100 (10.25%, 25/244) (Table S2 in Appendix B). Although the 2H2OP50 group can introduce the highest KI efficiency ( $P < 0.01$ ) (Fig. 2(A)), and 2H2OP system or 50 ng/ $\mu$ L of donors bring a significantly higher KI efficiency than the dsDNA method ( $P = 0.0001$ ) or 100 ng/ $\mu$ L of donors ( $P = 0.00469$ ) (Fig. 2(BC)). However, the dsDNA with 50 ng/ $\mu$ L donors demonstrated the highest on-target KI efficiency (10.80%, 23/213) compared to other treatments ( $P < 0.01$ ) (Fig. 2(D)). In contrast, only one on-target KI case was observed in the 2H2OP system, which was significantly lower than that in the dsDNA ( $P < 0.0001$ ) (Fig. 2(E)). Although different dosages of donors exhibited a significant effect on the total KI efficiency, our results indicated that this difference was not significant in the on-target KI ( $P = 0.3577$ ) (Fig. 2(F)).

480

481 According to the odds ratio, the 2H2OP system and low dosage tended to bear a  
482 higher total integrated rate which was 2.30 and 1.47 times than that of the dsDNA  
483 (OR = 2.30 for 2H2OP vs dsDNA) and high dosage (OR = 1.47 for 50 vs 100 ng/μL),  
484 respectively. Nonetheless, dsDNA had an overwhelming surpiority in on-target  
485 integration, which was more than 20 times greater than that in the 2H2OP system (OR  
486 = 26.70) (Table S3 in Appendix B). Taken together, the dsDNA system accompanied  
487 by a dosage of 50 ng/μL of donors tends to yield the highest on-target KI efficiency in  
488 our current study.

489

490 Given the non-As-Cath-integrated fish, we did detect individuals with only the LH  
491 mutation. Specifically, 5.56% (3/54), 6.67% (4/60), 3.33% (2/60), and 3.33% (2/60)  
492 of fish with *LH* deficiency in the 2H2OP50, 2H2OP100, dsDNA50 and dsDNA100  
493 groups, respectively, were detected by the Surveyor mutation test (Table S2 in  
494 Appendix B). The sequencing results revealed that 2, 2, 1 and 3 types of mutations in  
495 4 *LH*-mutant individuals from the 2H2OP100 group (Fig. S5 in Appendix B).

496

### 497 3.2. Effects of the dosage and CRISPR/Cas9 system

498

499 Different donor dosages and CRISPR/Cas9-mediated systems exhibited toxicity to  
500 fish embryos by decreasing the hatchability and fry survival rate. Although there were  
501 no significant differences in hatching rates among these four CRISPR/Cas9-mediated  
502 injected groups compared to the iCT group ( $P = 0.1630$ ), the hatching rate was lower  
503 than the nCT group ( $P < 0.01$ ) (Fig. 2(G)). Moreover, the lethality of embryos was  
504 consistent across different donor dosages (50 vs 100 ng/μL) ( $P = 0.1080$ ) or  
505 CRISPR/Cas9-mediated systems (2H2OP vs dsDNA) ( $P = 0.0796$ ), which was  
506 significantly higher than that in the nCT group (Fig. 2(HI)). For the fry survival, the  
507 survival rate of the microinjection group was significantly lower compared with the  
508 nCT group ( $P < 0.0001$ ) (Fig. 2(J)). In addition, the dsDNA system induced a higher  
509 survival rate of fry ( $P = 0.0031$ ) (Fig. 2(K)) than the 2H2OP system. Still, donor  
510 dosages showed no significant differences in fry survival after hatching ( $P = 0.2923$ )  
511 (Fig. 2(L)).

512

### 513 3.3. Mosaicism and As-Cath expression



514

515 PCR and RT-PCR were used to detect the *As-Cath* transgene and its expression of  
 516 different tissues in on-target positive fish. The results revealed that three of the five  
 517 LH<sup>-</sup>As-Cath<sup>+</sup> fish showed the expression of the *As-Cath* in all 14 sampled tissues  
 518 (skin, liver, kidney, spleen, blood, intestine, gill, stomach, fin, barbel, muscle, eye,  
 519 brain and gonad) (Fig. 3(AB)), but one of them had expression observed in 11 tissues  
 520 (except barbel, muscle and gill) and another one in 8 tissues (skin, liver, blood,  
 521 intestine, gill, barbel, muscle and gonad) (Fig. S6 in Appendix B), suggesting  
 522 mosaicism in the on-target positive individuals. We found that the expression of  
 523 *As-Cath* was detected even without pathogenic infections for the three on-target  
 524 positive individuals. The three highest mRNA levels were determined in the kidney  
 525 (28.91 fold changed), skin (24.30 fold), and gill (8.445 fold), followed by the muscle  
 526 (7.430 fold), spleen (6.047 fold) and barbel (4.808 fold). However, the eye (1.327  
 527 fold), intestine (1.589 fold), and fin (1.608 fold) had the lowest expression compared  
 528 to other tissues (Fig. 3(C)).

529

530 In addition, compared to the WT individuals, the mRNA level of *LH* in gonads was  
 531 down-regulated in LH<sup>-</sup>As-Cath<sup>+</sup> females at the age of one year ( $P = 0.0016$ ), but  
 532 there was no significant difference in that of males ( $P = 0.5817$ ) (Fig. 3(D)).

533

### 534 3.4. Reproductive sterility and restoration of reproduction

535

536 A three-round mating experiment determined the promise for complete control of  
 537 channel catfish reproduction (Fig. 4(A)). Our outcomes revealed that three pairs of  
 538 WT (100%, 7927 eggs/BW) and two pairs of LH<sup>+</sup>As-Cath<sup>+</sup> fish (50%, 8952  
 539 eggs/BW) were spawned respectively during the first two-week natural mating, but no  
 540 spawn was observed in the LH<sup>-</sup>As-Cath<sup>+</sup> pairs (0%). Compared to the LH<sup>-</sup>As-Cath<sup>+</sup>  
 541 pairs, WT and LH<sup>+</sup>As-Cath<sup>+</sup> fish had higher spawnability under natural pairing  
 542 conditions ( $P = 0.0148$  and  $P = 0.1743$ ). In addition, the LH<sup>+</sup>As-Cath<sup>+</sup> pairs did not  
 543 show a significant difference in spawnability compared to the WT pairs ( $P = 0.2143$ )  
 544 (Fig. 4(B)).

545

546 Furthermore, a one-week hormone priming (50 µg/kg LHRHa + 1600 IU/kg HCG) of  
 547 the males did not stimulate LH<sup>-</sup>As-Cath<sup>+</sup> females to give eggs, indicating

LH-deficient females blocked oocyte maturation and ovulation. However, our results discovered that a combination of LHRHa and HCG can effectively induce spawning for the LH<sup>-</sup>As-Cath<sup>+</sup> females when both males and females were primed. Specifically, two, two and one female gave eggs after 24 to 48 hours post-hormone injection from the 1200 IU (6213 eggs/BW), 1600 IU (5514 eggs/BW) and 2000 IU/kg (3778 eggs/BW) HCG group combined with 50 µg/kg LHRHa, respectively. These three treatments significantly improved the fecundity compared to 0.85 % NaCl injection ( $P < 0.0001$ ). Additionally, the fecundity decreased with increasing hormone dosage, but the difference among these three hormone dosages was not significant ( $P = 0.0731$ ). Nevertheless, the fecundity can be restored to a normal level when 1200 ( $P = 0.2627$ ) or 1600 ( $P = 0.1983$ ) IU/kg HCG combined with 50 µg/kg LHRHa was adopted (Fig. 4(C)). Compared with the WT and the other hormonal-therapy groups, the 2000 IU/kg HCG group significantly reduced the fecundity (3778 eggs/BW,  $P = 0.0494$ ) and hatchability (18.01%,  $P = 0.0476$ ) (Fig. 4(D)). Although different hormonal treatments had varying effects on fecundity and hatchability, they had no effects on fry survival at the early stage ( $P = 0.1018$ ) (Fig. 4(E)).

### 3.5. *F<sub>1</sub>* genotyping, growth comparison in *P<sub>0</sub>* and *F<sub>1</sub>*

As mentioned above, three WT, two LH<sup>+</sup>As-Cath<sup>+</sup>, and five LH<sup>-</sup>As-Cath<sup>+</sup> families were generated from our three-round mating experiment. However, genotype analysis determined that only one family in the LH<sup>+</sup>As-Cath<sup>+</sup> line (33.33% [10/30] integrated rate in the *F<sub>1</sub>* offspring) and two families in the LH<sup>-</sup>As-Cath<sup>+</sup> line (40% [12/30] integrated rate in the *F<sub>1</sub>* progeny of family 1 and 46.67% [14/30] integrated rate in the *F<sub>1</sub>* offspring of family 2), respectively, had the *As-Cath* gene detectable in the *F<sub>1</sub>* generation. These results further confirmed the existence of the mosaic phenomenon in the *P<sub>0</sub>* founders.

To determine the effects of *LH* disruption and *As-Cath* integration on fish growth, we compared the BW over time of the *P<sub>0</sub>* founders and the *F<sub>1</sub>* progeny, respectively. The growth data suggested that the LH<sup>-</sup>As-Cath<sup>+</sup> individuals did not show superiority in terms of growth in the first nine months in the *P<sub>0</sub>* generation. Nonetheless, *P<sub>0</sub>* LH<sup>-</sup>As-Cath<sup>+</sup> fish exhibited the largest body gain (36.35 g) compared to other genotypes (25 g). Furthermore, significantly faster growth was demonstrated in the *F<sub>1</sub>* generation of LH<sup>-</sup>As-Cath<sup>+</sup> after a three-month culture. Hence, our results indicated

583 more immediate growth potential for the LH<sup>-</sup>As-Cath<sup>+</sup> fish than the WT fish (Table  
584 2).

585

### 586 3.6. Enhanced resistance against fish pathogens

587

588 Enhanced resistance against *F. covae* and *E. ictaluri* of As-Cath-integrated fish was  
589 observed compared to WT/negative individuals from our challenge experiments in  
590 both P<sub>0</sub> and F<sub>1</sub> generations. According to *F. covae* challenge results, there was no  
591 significant difference in survival rate between the two types of controls (WT and  
592 LH<sup>+</sup>As-Cath<sup>-</sup>) in both P<sub>0</sub> (13.33% vs 20%, *P* = 0.8682) and F<sub>1</sub> generation (26.67% vs  
593 40%, *P* = 0.8955). However, LH<sup>-</sup>As-Cath<sup>+</sup> and LH<sup>+</sup>As-Cath<sup>+</sup> fish exhibited  
594 significantly improved survival post *F. covae* infection compared to the WT control  
595 group in both P<sub>0</sub> founders (LH<sup>-</sup>As-Cath<sup>+</sup> vs WT: 73.33% vs 13.33%, *P* = 0.0016;  
596 LH<sup>+</sup>As-Cath<sup>+</sup> vs WT: 66.67% vs 13.33%, *P* = 0.0014) and F<sub>1</sub> progeny  
597 (LH<sup>-</sup>As-Cath<sup>+</sup> vs WT: 86.67% vs 26.67%, *P* = 0.0010; LH<sup>+</sup>As-Cath<sup>+</sup> vs WT: 73.33%  
598 vs 26.67%, *P* = 0.0127). Additionally, on-target insertion of the *As-Cath* gene resulted  
599 in improved resistance against *F. covae* than in the off-target positives without  
600 statistically differing in both generations (73.33% vs 66.67%, *P* = 0.7726 for P<sub>0</sub>, and  
601 86.67% vs 73.33%, *P* = 0.3613 for F<sub>1</sub>). Furthermore, our findings revealed that the F<sub>1</sub>  
602 progeny was more resistant to *F. covae* than its P<sub>0</sub> parents (Fig. 5(AB)).

603

604 Increased resistance to *E. ictaluri* was also observed in the P<sub>0</sub> (LH<sup>-</sup>As-Cath<sup>+</sup> vs WT:  
605 73.33% vs 33.33%, *P* = 0.0125; LH<sup>+</sup>As-Cath<sup>+</sup> vs WT: 60% vs 33.33%, *P* = 0.0427)  
606 and F<sub>1</sub> generations (LH<sup>-</sup>As-Cath<sup>+</sup> vs WT: 66.67% vs 40%, *P* = 0.0558;  
607 LH<sup>+</sup>As-Cath<sup>+</sup> vs WT: 73.33% vs 40%, *P* = 0.0350), with results that were similar to  
608 those of the *F. covae* challenge. Overall, As-Cath-integrated individuals showed a  
609 significant improvement in the survival rate compared to the WT fish (66.67% vs  
610 33.33%, *P* = 0.0381 for P<sub>0</sub>; 70% vs 40%, *P* = 0.0335 for F<sub>1</sub>). Nevertheless, there was  
611 no significant difference in LH<sup>-</sup>As-Cath<sup>+</sup> and LH<sup>+</sup>As-Cath<sup>+</sup> fish (73.33% vs 60%, *P*  
612 = 0.4566 for P<sub>0</sub>; 66.67% vs 73.33%, *P* = 0.6851 for F<sub>1</sub>) (Fig. 5(CD)).

613

## 614 4. Discussion

615

616 In contrast to the previous gene-editing oriented exclusively to the improvement of  
617 the desired traits, the present study took into account ways to lessen the potential

impact of transgenic fish on the ecosystems and genetic biodiversity. Specifically, we successfully integrated an AMG into the reproduction-associated locus using different CRISPR/Cas9-mediated systems. We identified a suitable KI system for channel catfish to achieve boosted resistance against fish pathogens and reproductive control, reducing the reliance on antibiotics and anti-parasitics in aquaculture. The HA-mediated CRISPR/Cas9 system displayed a high integrated rate, low off-target events, and low toxicity. In addition, reproduction is entirely controllable and can only be restored to normal levels of fecundity with hormone therapy in the new fish line. In general, the insertion of the cathelicidin gene at the *LH* locus for enhanced resistance against infectious diseases and reproductive confinement to improve consumer-valued qualities and to promote the environmental friendliness of transgenic fish appears promising.

There have been several obstacles involved in the CRISPR/Cas9-mediated KI system when it is used in the embryos of non-model animals. In the history of genome editing, the initial CRISPR/Cas9 systems were proposed based on mammalian cells or embryos of the model animals. From model to non-model animals, there are several uncertainties, such as embryo size, developmental period, and the sensitivity to Cas9 protein that researchers have to optimize a fitted system when starting a new species' genome editing. Yoshimi et al. [5] demonstrated that the ssODN-mediated end joining approach induced a high integrated rate of 17.6% (3/17) in rats when a short ssODN template was provided. Conversely, recent works indicated that ssODN-mediated KI could induce a high percentage (17.8%) of indel mutations in sheep [41]. In the current study, we used CRISPR/Cas9 systems mediated by ssODN and HA to create on-target KIs of the *As-Cath* gene at the *LH* locus. Although a high KI efficiency of 22.38% (64/286) was detected in the ssODN-mediated system, it caused a high off-target frequency (> 90%) in the channel catfish. Our results are in agreement with findings in zebrafish, which have illustrated that erroneous ssODN integration occurred when various template lengths were adopted [7]. These studies suggest that ssODN-mediated KI efficiency in fish models relies heavily on ssODN templates [42], and caution is warranted when employing ssODNs to create KI models.

Compared to the ssODN-mediated system, HA-assisted KI can achieve a 20–30% HDR-mediated knockin in human cells with various homogenous sequences [9,43]. In addition, Simora et al. [44] determined that HA-mediated CRISPR/Cas9 provided

with a linear dsDNA donor displayed a total integrated rate of 29% at the non-coding region of channel catfish genome, which is drastically higher than that of this work (29% vs 11.16%[51/457]). We believe this difference in integration rate is due to the different sample sizes, unknown functions in the target regions (non-coding vs *LH* locus), efficiency of sgRNA and HA, and unpredictable genetic interaction; the larger sample size from our study could give more robust conclusions. These findings reveal that the HA-mediated system is more effective in the catfish species compared to the ssODN. The KI efficiency of HDR-induced CRISPR/Cas9 has been at a low level including in cell lines and model animals [5,7,9]. Fortunately, new CRISPR/Cas-mediated techniques are constantly being developed. For instance, the CRISPR/Cas12i-mediated system shows promise in multiplexed genome editing with high mutation rates in human T cells [45]. Additionally, Kelly et al. [46] established a CRISPR/Cas9 HITI system for the insertion of large DNA donors with high integrated efficiency of 36% in human 293T cells. Recently, a new approach named dCas9-SSAP demonstrated a high on-target KI efficiency (~20%) knocking in long sequences in mammalian cells [47]. These new tools or systems are encouraging to be applied from model to non-model animals and could improve genome-editing efficiency.

Although we predicted and avoided possible off-target sites using the well-acknowledged software, the actual integration results showed the existence of off-target activities. This is mainly due to the failure of *in silico* prediction to predict *bona-fide* off-target sites *in vivo* [48,49]. Furthermore, the frequency of off-target events is higher *in vivo* of animal experiments than that of in cellular experiments *in vitro* [50]. The majority of published studies contend that the observed unintended mutations/insertions is one major concern in the application of the CRISPR/Cas9 system, which could confound the interpretation of findings [49,51,52]. However, although some reports claim that no detectable undesirable mutations/insertions from the genotypes or phenotypes have been revealed in mice and fish [44,53,54], the following underlying potentials could be noted: 1) Unaltered phenotypes may be observed since the off-target cleavage can occur in a non-coding region [55]. 2) The researchers tend to focus on the P<sub>0</sub> founders with intended insertions rather than those harboring possible off-target mutations [56-57]. 3) Most published research using animal models does not use genome-wide methodologies for detecting off-target cases, which could conceal some infrequent off-target editing sites [50]. In the same case,

688 with the exception of *LH* mutations, we did not conduct a thorough detection on all  
689 off-target individuals due to its being time-consuming and expensive. Nevertheless,  
690 this does not preclude us from keeping the non-analyzed off-target individuals as we  
691 will eventually genotype them in a genome-wide and unbiased way.

692

693 Genetic mosaicisms have been and will still be another obstacle to applying  
694 CRISPR/Cas9-mediated genome editing in practical applications. In this study, we  
695 failed to effectively obtain a 100% of individuals without mosaics. In essence,  
696 mosaicism from CRISPR/Cas9-genome-edited organisms is common in the case of  
697 fertilized egg-based editing, and mosaic animals have been observed in mice [58,59],  
698 rats [57] and zebrafish [60,61] with a variety of frequencies. CRISPR/Cas9  
699 engineered mosaicisms bring undesired consequences, hindering the generation of  
700 homozygous positive offspring and prolonging the generation of homozygotes. We  
701 evaluated the *As-Cath* gene expression from five on-target positive P<sub>0</sub> founders and  
702 found that one individual had no expression in the gonad. In our study, several mosaic  
703 events were determined in the germline, resulting in the inability to transfer the  
704 *As-Cath* gene to the offspring. Thus, we believe that mosaicism is also common and  
705 unavoidable in non-model fish. Although early sperm/testis or egg/ovary genotyping  
706 can be effective in avoiding the creation of undesirable offspring, it is challenging to  
707 access the germline DNA without sacrificing the parents. Of importance, we still  
708 maintain our mosaic populations for genotyping and phenotyping in the further F<sub>2</sub> and  
709 F<sub>3</sub> progeny until homozygous individuals are obtained. Future research could reduce  
710 mosaicisms by delivering CRISPR/Cas9 components to very early-stage zygotes [6].  
711 Alternatively, the new strategies, i.e., *Easi*-CRISPR, C-CRISPR [6], CRISPR/Cas9  
712 HITI [46] and dCas9-SSAP [47] could be used to prevent the induction of mosaic  
713 animals.

714

715 Regardless of the type of CRISPR/Cas9-mediated genome editing, microinjection  
716 always has irreversible effects on embryos, i.e., increased mortality and decreased  
717 hatchability from our current study. High embryonic deaths were observed from  
718 shame- and CRISPR/Cas9-mediated-microinjection in our study, revealing that major  
719 mortality occurs due to the injection of the yolk, while fewer impacts are from the  
720 DNA donors and reagents [44]. Although a high dosage resulted in a high embryonic  
721 mortality and lower hatching rate, it did not significantly reduce the fry survival rate  
722 compared to the injected-control group, which is in agreement with the findings from

Elaswad et al. [35]. This may be because microinjection only has a detrimental effect on the yolk of the embryo. Still, this effect no longer affects the fry once the fertilized eggs have successfully hatched. Given the unavoidable physical lethality of embryos, off-target effects and mosaicisms, we recommend microinjection of ~3000 fertilized eggs for non-model fish species in order to afford enough gene-edited fish for subsequent validation experiments.

To assess the pleiotropic effects, we compared the growth performance of the on-target/off-target As-Cath-integrated fish line with the WT population. Our findings demonstrated that off-target insertions did not exhibit growth depression or improvement in various families of P<sub>0</sub> founders. Nonetheless, the preliminary data revealed the LH<sup>-</sup>As-Cath<sup>+</sup> fish had a greater gain in body weight compared to the WT individuals after a three-month culture in the tank, indicating that the growth differences are emerging in the F<sub>1</sub> progeny. This variation may be due to heterozygous individuals lacking stable genetic traits, or off-target integrations in other regions concealing growth advantages in the P<sub>0</sub> generation [50]. cfGnRH-deficient channel catfish did not show significant effects in growth and survival throughout a four-year culture compared to the WT fish [19]. However, potential pleiotropic effects could exist when the *LH* gene is replaced by the As-Cath in our cases. Therefore, P<sub>0</sub> mosaic founders carrying the *As-Cath* gene should be used to produce F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> homozygous families, and then the comparisons of the growth, survival rate, seinability and carcass traits could be performed to avail the enhanced performance of LH<sup>-</sup>As-Cath<sup>+</sup> fish line more transparent to farmers and the public in the future.

HDR-mediated KI is rarely applied in aquaculture due to the very low integration efficiency, but most of the traits were achieved by NHEJ-mediated KO [17,18]. In addition, few studies proved that gene-mutants can induce disease-resistant fish lines via KO to date [13]. By contrast, the integration of AMG is encouraging to improve resistance against pathogens in fish [13,16]. However, consumers generally have relatively little awareness of transgenesis and have more negative attitudes toward genetically modified organisms than genome-edited organisms [62], hence the public pushback against transgenic/gene-edited animals is hindering them from reaching the market. Here, we reasonably contend that cathelicidin transgenic catfish would not pose a threat to food safety since: 1) Meat from artificially grown alligators is edible



even when consumed raw, and the gut will digest most proteins and inactivate them. 2) Eventually, amino acids rather than proteins are absorbed by humans. 3) Even though the gene sequence is ever-changing in various beings, there are only 20 different types of encoded amino acids that are frequently consumed by humans. In this vein, we are raising attention of potential benefits and risks of our *As-Cath* transgenic catfish by making them transparent to the public.

Nonetheless, scientists and breeders need to be aware of the possible damage that genetically modified fish could cause to the environment and ecosystem [16]. On the one hand, reproductive sterility via genome editing has been attracting the attention of researchers and offering opportunities to reduce environmental risks in aquaculture [62]. On the other hand, representative examples have illustrated that reproductive confinement is promising in model and cultured fish by knocking out/disrupting gonadal development-related genes [23,63-66]. Recently, Qin et al. [19] demonstrated that the reproduction-blocked channel catfish are sterile, and this reproductive confinement can be lifted through hormone therapy with LHRHa. In this study, the dose of 1600 IU/kg HCG coupled with 50 µg/kg LHRHa can restore fecundity at the highest level in comparison to other hormone treatments, but this improvement is not significant from that of 1200 IU/kg HCG. Therefore, a low dose of 1200 IU/kg HCG is recommended for hormone therapy to restore the reproduction of the sterile fish line to reduce costs. In addition to genetically achieving reproductive sterility, well-confined culture systems should be adopted to avoid the escape of mutant/transgenic individuals, especially in the experimental phase of transgenic fish.

## 5. Conclusions

We established a sterile catfish line that confers enhanced resistance to fish pathogens by expressing the cathelicidin protein. Our study has demonstrated that the insertion of the cathelicidin gene at the *LH* locus by harnessing the HA- or ssODN-mediated CRISPR/Cas9 system can be a robust approach to produce sterilized and environmentally-sound fish lines with enhanced disease resistance. Encouragingly, CRISPR/Cas9-mediated KI of AMGs at the reproduction-related loci coupled with hormone therapy could be applied in other commercial fish to increase profits and lower environmental dangers posed by escaped genetic-modified individuals. Notably, even though the desired traits (on-target insertions) can be quickly achieved through

CRISPR/Cas9-mediated genome editing, this does not safeguard that we will be able to yield enough non-mosaic P<sub>0</sub> founders. We contend the genome-editing tool should be used as a complement to existing breeding techniques, not a replacement for them. Hence, a combination of genome editing and conventional selective breeding is required to maximize the benefits of CRISPR/Cas9 tools more effectively in aquatic applications and to hasten the breeding process. In conclusion, this study showed the potential of overexpressing a disease-resistant peptide inserted at a reproduction-related gene using CRISPR/Cas9 in channel catfish, which may provide a strategy of decreasing bacterial disease problems in catfish at the same time reducing environmental risks.

## Acknowledgments

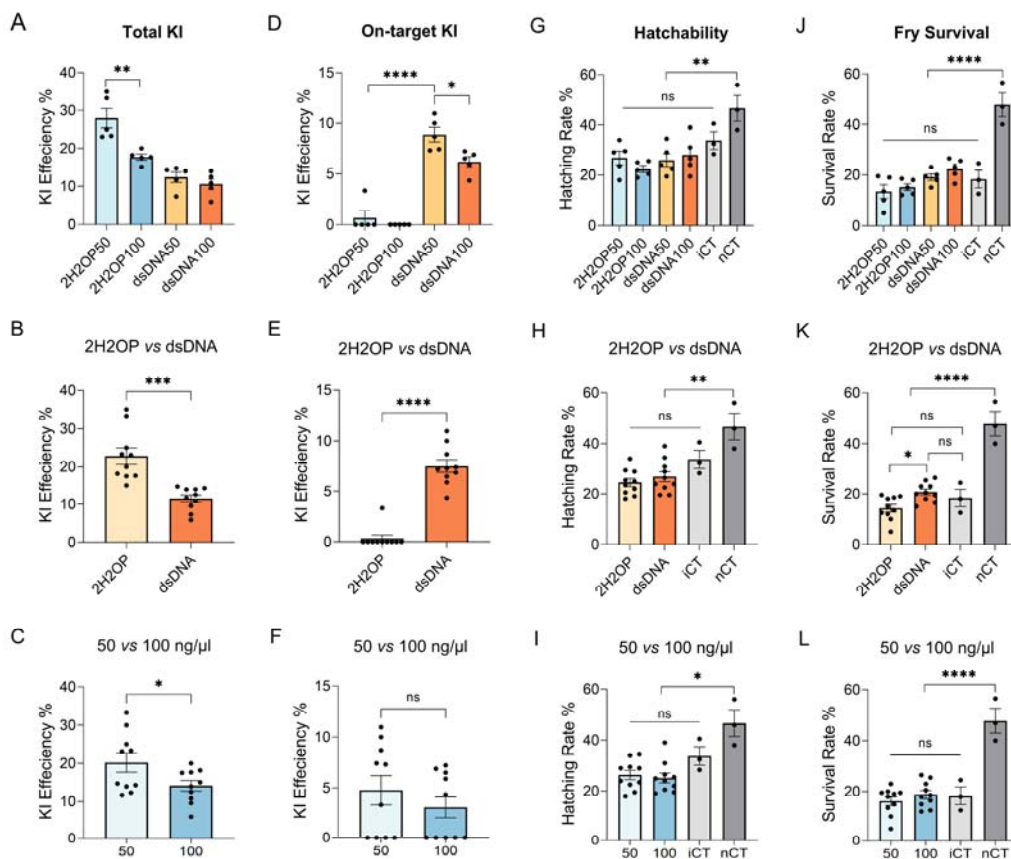
We thank Dr. Eric Peatman for providing the CFX96<sup>TM</sup> Real-Time System. This project was partially supported by USDA Grant No. G11941 (2018-33522-28769), and Alabama Agricultural Experiment Station grant (AAES-AIR). Jinhai Wang was supported by a scholarship from the China Scholarship Council.

## Appendix A. Supplementary data

Supplementary data to this article can be found from Appendix A and Appendix B.



region, and *As-Cath* gene region. (C) TAE agarose gel of PCR amplicons showing off-target positive detection of the ssODN1\_*As-Cath*\_ssODN2 construct using 2H2OP method. The promoter region (Prom-*As-CATH*, 519 bp) and *As-Cath* region (*As-CATH*-PolyA, 591 bp) were illustrated with sequencing results. (D) TAE agarose gel of PCR amplicons showing on-target positive detection of the HA1\_*As-Cath*\_HA2 construct using dsDNA method. The targeted gene regions (Prom-*As-CATH*, 542 bp and *As-CATH*-PolyA, 597 bp) and the junctional regions (HA1, 573 bp and HA2, 598 bp) were determined with sequencing results. The numbers on the top of the gel images indicate the sample IDs of the fish. Lane N, negative control using water as template; Lane W, wild-type control (nCT); Lane P, positive (plasmid or dsDNA donor) control; Lane M, DNA marker (1 kb), 500 and 650-bp bands are highlighted with black triangles; 50 and 100 ng/μL show the different doses of donors: plasmid or dsDNA.



865

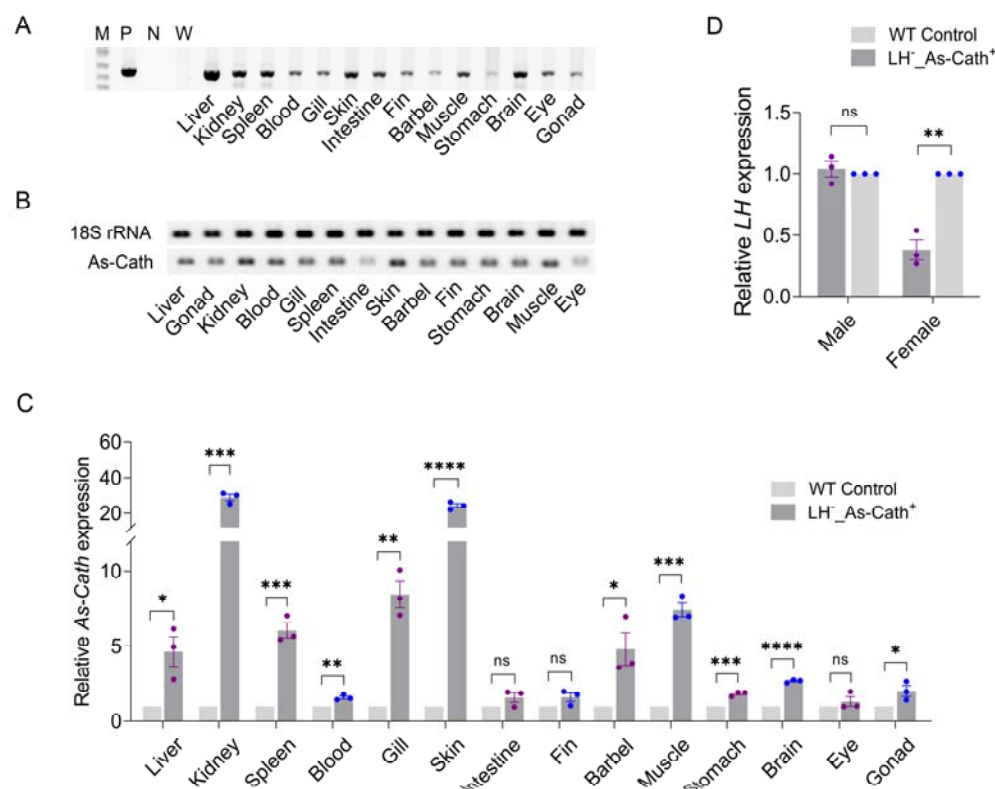
866 **Fig. 2. Effects of different CRISPR/Cas9-mediated systems (2H2OP vs dsDNA) with**  
867 **various dosages of donors (50 vs 100 ng/μL) on the knock-in (KI) efficiency, hatchability**  
868 **and fry survival rate. (A)** Total KI efficiency of different CRISPR/Cas9-mediated systems  
869 and dosage combinations. (B, C) Comparison of total KI efficiency for different systems or  
870 dosages of donors. (D) On-target KI efficiency of different CRISPR/Cas9-mediated systems  
871 and dosage combinations. (E, F) Comparison of on-target KI efficiency of different systems  
872 or dosages. (G) Effect of different CRISPR/Cas9-mediated systems and dosage combinations  
873 on hatchability. (H, I) Comparison of the hatchability for different systems or dosages. (J)  
874 Effect of different CRISPR/Cas9-mediated systems and dosage combinations on fry survival.

(K, L) Comparison of the fry survival rate for different systems or dosages. iCT, sham-injected control; nCT, non-injected control; 2H2OP(50/100), the CRISPR/Ca9-mediated system with ssODN1\_As-Cath\_ssODN2 construct (with a pUC57\_mini plasmid and ssODN donor as 50/100 ng/ $\mu$ L); dsDNA(50/100), the CRISPR/Ca9-mediated system with HA1\_As-Cath\_HA2 donor DNA (with a dsDNA donor as 50/100 ng/ $\mu$ L); \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ; \*\*\*\* =  $P < 0.0001$ ; ns = not significant, by unpaired student's  $t$ -test or one-way ANOVA.

882

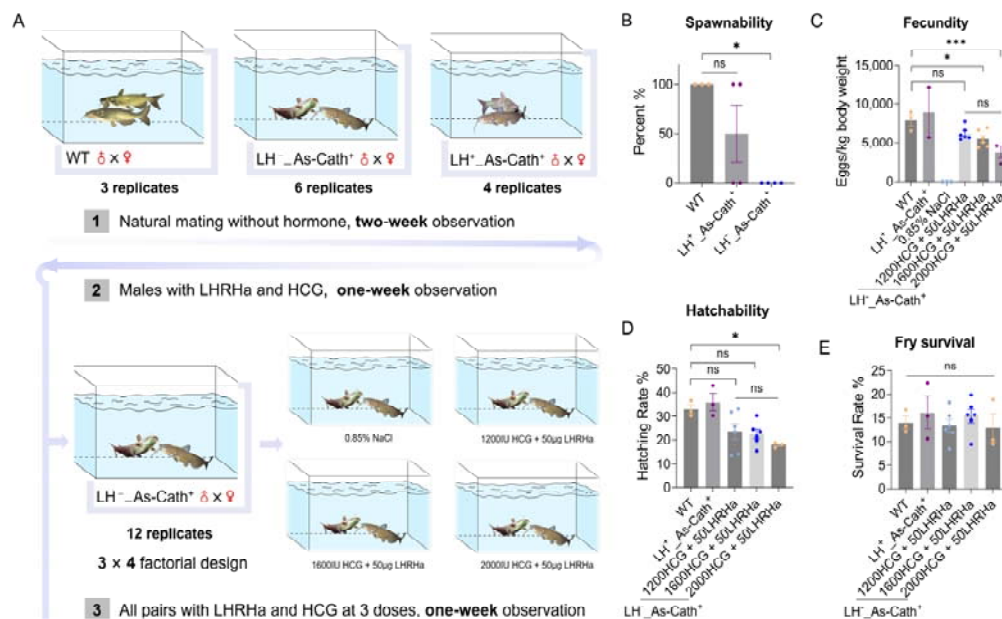
883

884



**Fig. 3. Mosaicism detection and the expression of the cathelicidin gene from *Alligator sinensis* (*As-Cath*) in the  $LH^-As-Cath^+$  fish line.** (A) PCR amplicons show the *As-Cath* region in 14 tissues from one representative  $LH^-As-Cath^+$  fish. (B) The agarose gel electrophoresis showed the *As-Cath* gene expression in various tissues of  $P_0$  transgenic channel catfish, *Ictalurus punctatus*. (C) Relative *As-Cath* gene expression of different tissues from RT-PCR analyses. (D) Relative *LH* gene expression of gonads from  $LH^-As-Cath^+$  males and females. Expression levels were calibrated against corresponding tissues from sibling wild-type fish, and three individuals were employed for each genotype. Lane M, DNA marker (1 kb); Lane P, positive (plasmid or dsDNA donor) control; Lane N, water negative control; Lane W, wild-type control (nCT); \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ; \*\*\*\* =  $P < 0.0001$ ; ns = not significant, by unpaired student's  $t$ -test or one-way ANOVA.

886

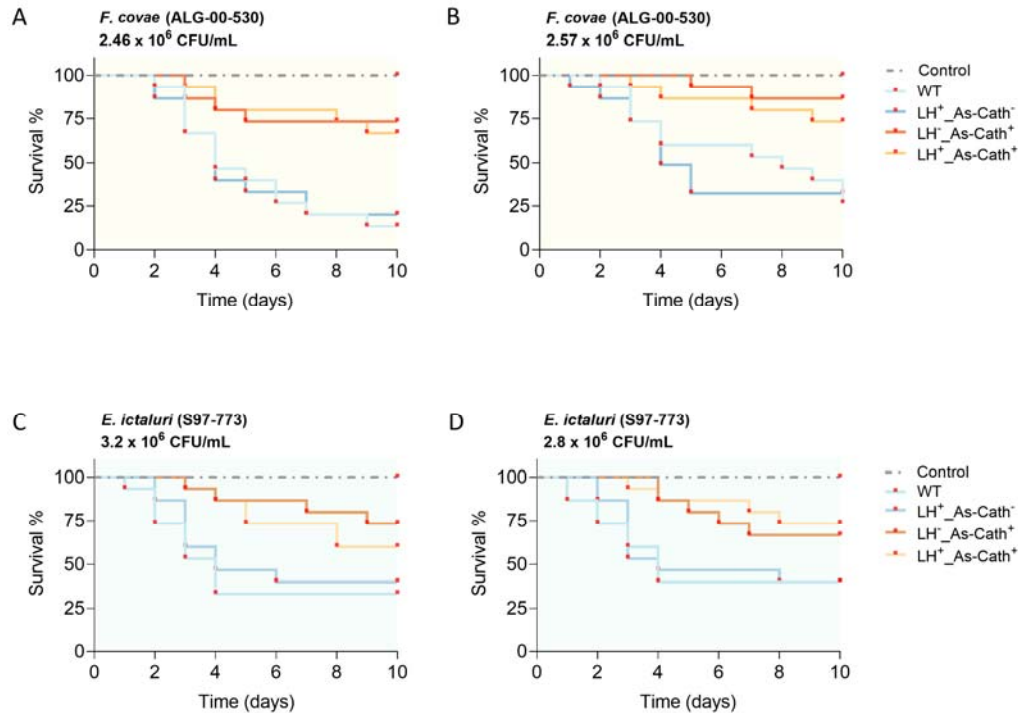


**Fig. 4. Reproductive determination and restoration of the As-Cath-integrated fish lines.**

(A) A three-round design of the reproduction experiment. Three genotypes of P<sub>0</sub> founders: WT, LH<sup>-</sup>As-Cath<sup>+</sup>, and LH<sup>+</sup>As-Cath<sup>+</sup> fish were involved. First round, 3, 6 and 4 pairs as replicates for each genotype were set up randomly in 13 tanks for mating without hormone treatments, and a two-week observation was adopted. Second round, moved out spawned pairs and primed un-mated males with a 50 µg/kg LHRHa implant and 1600 IU/kg HCG to determine the reproduction of LH<sup>-</sup>As-Cath<sup>+</sup> females, observing for one week. Third round, 12 pairs of LH<sup>-</sup>As-Cath<sup>+</sup> fish were complemented and re-paired and treated with three doses of LHRHa and HCG in a 3 × 4 factorial design for one week. (B) Detection of spawnability for LH<sup>-</sup>As-Cath<sup>+</sup> fish during natural mating. (C, D, E) Potential effects of different hormone treatments on the fecundity and hatchability of P<sub>0</sub> generation, and fry survival of F<sub>1</sub> generation. LH, luteinizing hormone; LHRHa, luteinizing hormone-releasing hormone analogue; HCG, human chorionic gonadotropin; \* = *P* < 0.05; \*\* = *P* < 0.01; ns = not significant, by unpaired student's *t*-test or one-way ANOVA.



922



923

924 **Fig. 5. Kaplan-Meier plots of *As-Cath* integrated catfish against two fish bacterial**  
 925 **pathogens. (A, B) Survival curves of P<sub>0</sub> and F<sub>1</sub> generations for a variety of genotypes**  
 926 **infected by *Flavobacterium covaе*, respectively. (C, D) Survival curves of P<sub>0</sub> founders and F<sub>1</sub>**  
 927 **progeny for different genotypes infected by *Edwardsiella ictaluri*, respectively. In addition to**  
 928 **these bacterial infection groups, one control group with medium immersion was implanted for**  
 929 **each challenge experiment, and the immersion dose was presented in each figure. Comparison**  
 930 **of different survival curves was determined by the Log-rank (Mantel-Cox) test. WT,**  
 931 **wild-type, non-injected fish line; LH<sup>+</sup>As-Cath<sup>-</sup>, negative fish line (micro-injected fish**  
 932 **without *LH* mutation and *As-Cath* insertion); LH<sup>-</sup>As-Cath<sup>+</sup>, on-target positive fish (*As-Cath***  
 933 **insertion was detected at *LH* locus); LH<sup>+</sup>As-Cath<sup>+</sup>, off-target positive fish (*As-Cath* insertion**  
 934 **was detected but not at *LH* locus).**



**Table 1.** Target sequences of sgRNAs and the universal primer used in the present study. Underlined sequences represent the protospacer adjacent motif.

sgRNA	Targeted sequence for sgRNA (5'-3')
sgRNA1	5'- TTCAAACCGCCATCTGCAGC <u>GGG</u> -3'
sgRNA2	5'- GCGGACAGGTATCCGGTAAG <u>CGG</u> -3'
Universal primer	TTTTGCACCGACTCGGTGCCACTTTTCAAGTTGATAACGGACTAG CCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

**Table 2.** Mean monthly body weight (BW), sample size (N) over time of P<sub>0</sub> and F<sub>1</sub> As-Cath-integrated, negative and control channel catfish, *Ictalurus punctatus*. P<sub>0</sub> founders were generated in June 2020, and F<sub>1</sub> progeny were produced in June 2022. For both generations, four genotypes: WT, LH<sup>+</sup>As-Cath<sup>-</sup>, LH<sup>-</sup>As-Cath<sup>+</sup>, and LH<sup>+</sup>As-Cath<sup>+</sup> were included. Fish were kept separately in 60-L aquaria with the density of 2 fry/L until 4 months post hatch, then they were pit-tagged (10/2/2020) and transferred to a 1,200-L circular tank (~800-L water) with a mix of these 4 genotypes (initial number of fish was 30, 30, 28 and 32) and fed daily to satiation. Differences in BW among these four genotypes were compared using one-way ANOVA followed by Tukey's multiple comparisons test. Means with different letters as superscripts are significantly different ( $P < 0.05$ ).

Genotype		Mean body weight (g) of fish at different ages (Mean $\pm$ SEM)									
		10/2/2020		11/14/2020		12/14/2020		1/25/2021		3/6/2021	
		BW	N	BW	N	BW	N	BW	N	BW	N
<b>P<sub>0</sub></b>	WT	27.20 $\pm$ 1.77 <sup>a</sup>	60	37.15 $\pm$ 2.83 <sup>a</sup>	30	42.45 $\pm$ 3.08 <sup>ab</sup>	30	36.75 $\pm$ 2.31 <sup>a</sup>	30	50.75 $\pm$ 3.58 <sup>a</sup>	27
	LH <sup>+</sup> As-Cath <sup>-</sup>	26.30 $\pm$ 2.24 <sup>a</sup>	60	36.40 $\pm$ 2.14 <sup>a</sup>	30	38.30 $\pm$ 3.20 <sup>a</sup>	29	35.25 $\pm$ 3.18 <sup>a</sup>	29	51.10 $\pm$ 2.28 <sup>a</sup>	29
	LH <sup>-</sup> As-Cath <sup>+</sup>	23.10 $\pm$ 1.72 <sup>a</sup>	41	41.30 $\pm$ 2.60 <sup>a</sup>	28	49.65 $\pm$ 2.35 <sup>b</sup>	21	43.20 $\pm$ 2.75 <sup>a</sup>	20	58.45 $\pm$ 4.21 <sup>a</sup>	20
	LH <sup>+</sup> As-Cath <sup>+</sup>	27.75 $\pm$ 2.39 <sup>a</sup>	63	39.95 $\pm$ 2.73 <sup>a</sup>	32	47.25 $\pm$ 3.26 <sup>ab</sup>	33	34.50 $\pm$ 3.58 <sup>a</sup>	33	50.85 $\pm$ 2.89 <sup>a</sup>	33
<b>F<sub>1</sub></b>	8/9/2022			9/11/2022		10/12/2022					
		BW	N	BW	N	BW	N				
	WT	2.63 $\pm$ 0.16 <sup>a</sup>	60	15.13 $\pm$ 1.00 <sup>a</sup>	54	22.90 $\pm$ 1.23 <sup>a</sup>	54				
	LH <sup>+</sup> As-Cath <sup>-</sup>	2.60 $\pm$ 0.16 <sup>a</sup>	60	14.67 $\pm$ 0.91 <sup>a</sup>	56	21.30 $\pm$ 1.03 <sup>a</sup>	54				
	LH <sup>-</sup> As-Cath <sup>+</sup>	3.03 $\pm$ 0.14 <sup>a</sup>	60	19.57 $\pm$ 1.31 <sup>b</sup>	59	26.03 $\pm$ 1.32 <sup>b</sup>	57				
	LH <sup>+</sup> As-Cath <sup>+</sup>	2.70 $\pm$ 0.12 <sup>a</sup>	60	13.14 $\pm$ 1.05 <sup>a</sup>	58	22.13 $\pm$ 1.09 <sup>a</sup>	58				

WT, wild-type fish without injection; LH<sup>+</sup>As-Cath<sup>-</sup>, negative fish without the *As-Cath* insertion or *LH* gene mutation; LH<sup>-</sup>As-Cath<sup>+</sup>, on-target positive fish with the integration of the *As-Cath* gene at the *LH* locus; LH<sup>+</sup>As-Cath<sup>+</sup>, off-target positive fish with the *As-Cath* insertion but no *LH* mutation.

## 963      **References**

- 964      [1] Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9.  
965              Science 2014;346:1258096. <https://doi.org/10.1126/science.1258096>.
- 966      [2] Storici F, Snipe JR, Chan GK, Gordenin DA, Resnick MA. Conservative repair of a  
967              chromosomal double-strand break by single-strand DNA through two steps of annealing.  
968              Mol Cell Biol 2006;26:7645–57. <https://doi.org/10.1128/mcb.00672-06>.
- 969      [3] Chen F, Pruett-Miller SM, Huang Y, Gjoka M, Duda K, Taunton J, et al. High-frequency  
970              genome editing using ssDNA oligonucleotides with zinc-finger nucleases. Nat Methods  
971              2011;8:753–5. <https://doi.org/10.1038/nmeth.1653>.
- 972      [4] Wefers B, Meyer M, Ortiz O, Hrabé de Angelis M, Hansen J, Wurst W, et al. Direct  
973              production of mouse disease models by embryo microinjection of TALENs and  
974              oligodeoxynucleotides. Proc Natl Acad Sci USA 2013;110:3782–87.  
975              <https://doi.org/10.1073/pnas.1218721110>.
- 976      [5] Yoshimi K, Kunihiro Y, Kaneko T, Nagahora H, Voigt B, Mashimo T. ssODN-mediated  
977              knock-in with CRISPR-Cas for large genomic regions in zygotes. Nat Commun  
978              2016;7:10431. <https://doi.org/10.1038/ncomms10431>.
- 979      [6] Mehravara M, Shirazia A, Nazaric M, Banand M. Mosaicism in CRISPR/Cas9-mediated  
980              genome editing. Dev Biol 2019;445:156–62.  
981              <https://doi.org/10.1016/j.ydbio.2018.10.008>.
- 982      [7] Boel A, De Saffel H, Steyaert W, Callewaert B, De Paepe A, Coucke PJ, et al.  
983              CRISPR/Cas9-mediated homology-directed repair by ssODNs in zebrafish induces  
984              complex mutational patterns resulting from genomic integration of repair-template  
985              fragments. Dis Model Mech 2018;11:dmm035352.  
986              <https://doi.org/10.1242/dmm.035352>.
- 987      [8] Hisano Y, Sakuma T, Nakade S, Ohga R, Ota S, Okamoto H, et al. Precise in-frame  
988              integration of exogenous DNA mediated by CRISPR/Cas9 system in zebrafish. Sci Rep  
989              2015;5:8841. <https://doi.org/10.1038/srep08841>.
- 990      [9] Zhang J-P, Li X-L, Li G-H, Chen W, Arakaki C, Botimer GD, et al. Efficient precise  
991              knockin with a double cut HDR donor after CRISPR/Cas9-mediated double-stranded  
992              DNA cleavage. Genome Biology 2017;18. <https://doi.org/10.1186/s13059-017-1164-8>.
- 993      [10] Murakami Y, Ansai S, Yonemura A, Kinoshita M. An efficient system for  
994              homology-dependent targeted gene integration in medaka (*Oryzias latipes*). Zoological  
995              Lett 2017;3:10. <https://doi.org/10.1186/s40851-017-0071-x>.

- 996 [11] Ledford H. Salmon approval heralds rethink of transgenic animals. Nature  
997 2015;527:417–18. <https://doi.org/10.1038/527417a>.
- 998 [12] Waltz E. First genetically engineered salmon sold in Canada. Nature 2017;548:148–48.  
999 <https://doi.org/10.1038/nature.2017.22116>.
- 1000 [13] Wang J, Su B, Dunham RA. Genome-wide identification of catfish antimicrobial  
1001 peptides: A new perspective to enhance fish disease resistance. Rev Aquac  
1002 2022a;14:2002–22. <https://doi.org/10.1111/raq.12684>.
- 1003 [14] Xing D, Su B, Bangs M, Li S, Wang J, Bern L, et al. CRISPR/Cas9-mediate knock-in  
1004 method can improve the expression and effect of transgene in P1 generation of channel  
1005 catfish (*Ictalurus punctatus*). Aquaculture. 2022a;560:738531.  
1006 <https://doi.org/10.1016/j.aquaculture.2022.738531>.
- 1007 [15] Xing D, Su B, Li S, Bangs M, Creamer D, Coogan M, et al. CRISPR/Cas9-mediated  
1008 transgenesis of the Masu salmon (*Oncorhynchus masou*) *elovl2* gene improves n-3 fatty  
1009 acid content in channel catfish (*Ictalurus punctatus*). Mar Biotechnol 2022b;24:513–23.  
1010 <https://doi.org/10.1007/s10126-022-10110-6>.
- 1011 [16] Dunham RA, Su B. Genetically Engineered Fish: Potential Impacts on Aquaculture,  
1012 Biodiversity, and the Environment. In: Chaurasia A, Hawksworth DL, Pessoa de  
1013 Miranda M, editors. GMOs: Implications for Biodiversity Conservation and Ecological  
1014 Processes, Cham: Springer International Publishing; 2020, p. 241–75.  
1015 [https://doi.org/10.1007/978-3-030-53183-6\\_11](https://doi.org/10.1007/978-3-030-53183-6_11).
- 1016 [17] Blix TB, Dalmo RA, Wargelius A, Myhr AI. Genome editing on finfish: Current status  
1017 and implications for sustainability. Rev Aquac 2021;13:2344–63.  
1018 <https://doi.org/10.1111/raq.12571>.
- 1019 [18] Yang Z, Yu Y, Tay YX, Yue GH. Genome editing and its applications in genetic  
1020 improvement in aquaculture. Rev Aquac 2022;14:178–91.  
1021 <https://doi.org/10.1111/raq.12591>.
- 1022 [19] Qin G, Qin Z, Lu C, Ye Z, Elawad A, Bangs M, et al. Gene editing of the catfish  
1023 gonadotropin-releasing hormone gene and hormone therapy to control the reproduction  
1024 in channel catfish, *Ictalurus punctatus*. Biology (Basel) 2022;11:649.  
1025 <https://doi.org/10.3390/biology11050649>.
- 1026 [20] Grier HJ. Cellular organization of the testis and spermatogenesis in fishes. Am Zool  
1027 1981;21:345–57. <https://doi.org/10.1093/icb/21.2.345>.
- 1028 [21] Yamaguchi Y, Nagata J, Nishimiya O, Kawasaki T, Hiramatsu N, Todo T. Molecular  
1029 characterization of *fshb* and *lhb* subunits and their expression profiles in captive

- 1030 white-edged rockfish, *Sebastes taczanowskii*. Comp Biochem Physiol A: Mol Integr  
1031 Physiol 2021;261:111055. <https://doi.org/10.1016/j.cbpa.2021.111055>.
- 1032 [22] Chu L, Li J, Liu Y, Hu W, Cheng CHK. Targeted gene disruption in zebrafish reveals  
1033 noncanonical functions of LH signaling in reproduction. Mol Endocrinol  
1034 2014;28:1785–95. <https://doi.org/10.1210/me.2014-1061>.
- 1035 [23] Qin Z, Li Y, Su B, Cheng Q, Ye Z, Perera DA, et al. Editing of the luteinizing hormone  
1036 gene to sterilize channel catfish, *Ictalurus punctatus*, using a modified zinc finger  
1037 nuclease technology with electroporation. Mar Biotechnol 2016;18:255–63.  
1038 <https://doi.org/10.1007/s10126-016-9687-7>.
- 1039 [24] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell  
1040 2006;124:783–801. <https://doi.org/10.1016/j.cell.2006.02.015>.
- 1041 [25] Wang G, Li X, Wang Z. APD3: the antimicrobial peptide database as a tool for research  
1042 and education. Nucleic Acids Res 2016;44:D1087–93.  
1043 <https://doi.org/10.1093/nar/gkv1278>.
- 1044 [26] Wang J, Wilson AE, Su B, Dunham RA. Functionality of dietary antimicrobial peptides  
1045 in aquatic animal health: Multiple meta-analyses. Anim Nutr 2022b.  
1046 <https://doi.org/10.1016/j.aninu.2022.10.001>.
- 1047 [27] Mookherjee N, Anderson MA, Haagsman HP, Davidson DJ. Antimicrobial host defence  
1048 peptides: functions and clinical potential. Nat Rev Drug Discov 2020;19:311–32.  
1049 <https://doi.org/10.1038/s41573-019-0058-8>.
- 1050 [28] Hilchie AL, Wuerth K, Hancock REW. Immune modulation by multifaceted cationic  
1051 host defense (antimicrobial) peptides. Nat Chem Biol 2013;9:761–68.  
1052 <https://doi.org/10.1038/nchembio.1393>.
- 1053 [29] Chen Y, Cai S, Qiao X, Wu M, Guo Z, Wang R, et al. As-CATH1-6, novel cathelicidins  
1054 with potent antimicrobial and immunomodulatory properties from *Alligator sinensis*,  
1055 play pivotal roles in host antimicrobial immune responses. Biochem J  
1056 2017;474:2861–85. <https://doi.org/10.1042/BCJ20170334>.
- 1057 [30] Simora RMC, Li S, Abass NY, Terhune JS, Dunham RA. Cathelicidins enhance  
1058 protection of channel catfish, *Ictalurus punctatus*, and channel catfish ♀ × blue catfish,  
1059 *Ictalurus furcatus* ♂ hybrid catfish against *Edwardsiella ictaluri* infection. J Fish Dis  
1060 2020;43:1553–62. <https://doi.org/10.1111/jfd.13257>.
- 1061 [31] Simora RMC, Wang W, Coogan M, El Hussein N, Terhune JS, Dunham RA.  
1062 Effectiveness of cathelicidin antimicrobial peptide against Ictalurid catfish bacterial  
1063 pathogens. J Aquat Anim Health 2021;33:178–89. <https://doi.org/10.1002/aah.10131>.

- 1064 [32] Liu Z, Liu S, Yao J, Bao L, Zhang J, Li Y, et al. The channel catfish genome sequence  
1065 provides insights into the evolution of scale formation in teleosts. Nat Commun  
1066 2016;7:11757. <https://doi.org/10.1038/ncomms11757>.
- 1067 [33] Mosimann C, Kaufman CK, Li P, Pugach EK, Tamplin OJ, Zon LI. Ubiquitous  
1068 transgene expression and Cre-based recombination driven by the ubiquitin promoter in  
1069 zebrafish. Development 2011;138:169–77. <https://doi.org/10.1242/dev.059345>.
- 1070 [34] Bae S, Park J, Kim J-S. Cas-OFFinder: a fast and versatile algorithm that searches for  
1071 potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics  
1072 2014;30:1473–75. <https://doi.org/10.1093/bioinformatics/btu048>.
- 1073 [35] Elaswad A, Khalil K, Ye Z, Liu Z, Liu S, Peatman E, et al. Effects of CRISPR/Cas9  
1074 dosage on TICAM1 and RBL gene mutation rate, embryonic development, hatchability  
1075 and fry survival in channel catfish. Sci Rep 2018;8:16499.  
1076 <https://doi.org/10.1038/s41598-018-34738-4>.
- 1077 [36] Khalil K, Elayat M, Khalifa E, Daghash S, Elaswad A, Miller M, et al. Generation of  
1078 myostatin gene-edited channel catfish (*Ictalurus punctatus*) via zygote injection of  
1079 CRISPR/Cas9 system. Sci Rep 2017;7:7301.  
1080 <https://doi.org/10.1038/s41598-017-07223-7>.
- 1081 [37] Armstrong JB, Malacinski GM. Developmental Biology of the Axolotl. New York:  
1082 Oxford University Press; 1989.
- 1083 [38] Qiu P, Shandilya H, D'Alessio JM, O'Connor K, Durocher J, Gerard GF. Mutation  
1084 detection using Surveyor<sup>TM</sup> nuclease. Biotechniques 2004;36:702–7.  
1085 <https://doi.org/10.2144/04364PF01>.
- 1086 [39] Coogan M, Alston V, Su B, Khalil K, Elaswad A, Khan M, et al. CRISPR/Cas-9 induced  
1087 knockout of myostatin gene improves growth and disease resistance in channel catfish  
1088 (*Ictalurus punctatus*). Aquaculture 2022;557:738290.  
1089 <https://doi.org/10.1016/j.aquaculture.2022.738290>.
- 1090 [40] Davis KB. Age at puberty of channel catfish, *Ictalurus punctatus*, controlled by  
1091 thermoperiod. Aquaculture 2009;292:244–47.  
1092 <https://doi.org/10.1016/j.aquaculture.2009.04.023>.
- 1093 [41] Menchaca A, Dos Santos-Neto PC, Souza-Neves M, Cuadro F, Mulet AP, Tesson L, et al.  
1094 Otoferrin gene editing in sheep via CRISPR-assisted ssODN-mediated homology  
1095 directed repair. Sci Rep 2020;10:5995. <https://doi.org/10.1038/s41598-020-62879-y>.

- 1096 [42] Kan Y, Ruis B, Takasugi T, Hendrickson EA. Mechanisms of precise genome editing  
1097 using oligonucleotide donors. *Genome Res* 2017;27:1099–111.  
1098 <https://doi.org/10.1101/gr.214775.116>.
- 1099 [43] Byrne SM, Ortiz L, Mali P, Aach J, Church GM. Multi-kilobase homozygous targeted  
1100 gene replacement in human induced pluripotent stem cells. *Nucleic Acids Res*  
1101 2015;43:e21. <https://doi.org/10.1093/nar/gku1246>.
- 1102 [44] Simora RMC, Xing D, Bangs MR, Wang W, Ma X, Su B, et al.  
1103 CRISPR/Cas9-mediated knock-in of alligator cathelicidin gene in a non-coding  
1104 region of channel catfish genome. *Sci Rep* 2020;10:22271.  
1105 <https://doi.org/10.1038/s41598-020-79409-5>.
- 1106 [45] McGaw C, Garrity AJ, Munoz GZ, Haswell JR, Sengupta S, Keston-Smith E, et al.  
1107 Engineered Cas12i2 is a versatile high-efficiency platform for therapeutic genome  
1108 editing. *Nat Commun* 2022;13:2833 <https://doi.org/10.1038/s41467-022-30465-7>.
- 1109 [46] Kelly JJ, Saeed-Marand M, Nyström NN, Evans MM, Chen Y, Martinez FM, et al. Safe  
1110 harbor-targeted CRISPR-Cas9 homology-independent targeted integration for  
1111 multimodality reporter gene-based cell tracking. *Sci Adv* 2021;7: eabc3791.  
1112 <https://doi.org/10.1126/sciadv.abc3791>
- 1113 [47] Wang C, Qu Y, Cheng JKW, Hughes NW, Zhang Q, Wang M, et al. dCas9-based gene  
1114 editing for cleavage-free genomic knock-in of long sequences. *Nat Cell Biol*  
1115 2022;24:268–78. <https://doi.org/10.1038/s41556-021-00836-1>.
- 1116 [48] Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using  
1117 the CRISPR-Cas9 system. *Nat Protoc* 2013;8:2281–308.  
1118 <https://doi.org/10.1038/nprot.2013.143>.
- 1119 [49] Heigwer F, Kerr G, Boutros M. E-CRISP: fast CRISPR target site identification. *Nat*  
1120 *Methods* 2014;11:122–23. <https://doi.org/10.1038/nmeth.2812>.
- 1121 [50] Zhang X-H, Tee LY, Wang X-G, Huang Q-S, Yang S-H. Off-target effects in  
1122 CRISPR/Cas9-mediated genome engineering. *Mol Ther Nucleic Acids* 2015;17:e264.  
1123 <https://doi.org/10.1038/mtna.2015.37>.
- 1124 [51] Pattanayak V, Lin S, Guilinger JP, Ma E, Doudna JA, Liu DR. High-throughput profiling  
1125 of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat*  
1126 *Biotechnol* 2013;31:839–43. <https://doi.org/10.1038/nbt.2673>.
- 1127 [52] Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S, et al. Analysis of off-target effects of  
1128 CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res*  
1129 2014;24:132–41. <https://doi.org/10.1101/gr.162339.113>.



- 1130 [53] Shen B, Zhang J, Wu H, Wang J, Ma K, Li Z, et al. Generation of gene-modified mice  
1131 via Cas9/RNA-mediated gene targeting. *Cell Res* 2013;23:720–23.  
1132 <https://doi.org/10.1038/cr.2013.46>
- 1133 [54] Iyer V, Shen B, Zhang W, Hodgkins A, Keane T, Huang X, et al. Off-target mutations  
1134 are rare in Cas9-modified mice. *Nat Methods* 2015;12:479.  
1135 <https://doi.org/10.1038/nmeth.3408>.
- 1136 [55] Wang T, Wei JJ, Sabatini DM, Lander, ES. Genetic screens in human cells using the  
1137 CRISPR-Cas9 system. *Science* 2014;343:80–84.  
1138 <https://doi.org/10.1126/science.1246981>.
- 1139 [56] Li D, Qiu Z, Shao Y, Chen Y, Guan Y, Liu M, et al. Heritable gene targeting in the  
1140 mouse and rat using a CRISPR-Cas system. *Nat Biotechnol* 2013;31:681–83.  
1141 <https://doi.org/10.1038/nbt.2661>.
- 1142 [57] Li W, Teng F, Li T, Zhou Q. Simultaneous generation and germline transmission of  
1143 multiple gene mutations in rat using CRISPR-Cas systems. *Nat Biotechnol* 2013;31:684.  
1144 <https://doi.org/10.1038/nbt.2652>.
- 1145 [58] Oliver D, Yuan S, McSwiggin H, Yan W. Pervasive genotypic mosaicism in founder  
1146 mice derived from genome editing through pronuclear injection. *PLOS One* 2015;10:  
1147 e0129457. <https://doi.org/10.1371/journal.pone.0129457>.
- 1148 [59] Raveux A, Vandormael-Pournin S, Cohen-Tannoudji M. Optimization of the production  
1149 of knock-in alleles by CRISPR/Cas9 microinjection into the mouse zygote. *Sci Rep*  
1150 2017;7:42661. <https://doi.org/10.1038/srep42661>.
- 1151 [60] Jao L-E, Wente SR, Chen W. Efficient multiplex biallelic zebrafish genome editing  
1152 using a CRISPR nuclease system. *Proc Natl Acad Sci USA* 2013;110:13904–909.  
1153 <https://doi.org/10.1073/pnas.1308335110>.
- 1154 [61] Auer TO, Duroure K, De Cian A, Concordet JP, Del Bene F. Highly efficient  
1155 CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair.  
1156 *Genome Res* 2014;24:142–53. <https://doi.org/10.1101/gr.161638.113>.
- 1157 [62] Hallerman EM, Dunham R, Houston RD, Walton M, Wargelius A, Wray-Cahen D.  
1158 Towards production of genome-edited aquaculture species. *Rev Aquac* 2022;1–5.  
1159 <https://doi.org/10.1111/raq.12739>.
- 1160 [63] Wargelius A, Leininger S, Skaftnesmo KO, Kleppe L, Andersson E, Taranger GL, et al.  
1161 *Dnd* knockout ablates germ cells and demonstrates germ cell independent sex  
1162 differentiation in Atlantic salmon. *Sci Rep*. 2016;6:21284.  
1163 <https://doi.org/10.1038/srep21284>.

- 1164 [64] Gay S, Bugeon J, Bouchareb A, Henry L, Delahaye C, Legeai F, et al. MiR-202 controls  
1165 female fecundity by regulating medaka oogenesis. PLoS Genet 2018;14:e1007593.  
1166 <https://doi.org/10.1371/journal.pgen.1007593>.
- 1167 [65] Su B, Peatman E, Shang M, Thresher R, Grewe P, Patil JG, et al. Expression and  
1168 knockdown of primordial germ cell genes, *vasa*, *nanos* and *dead end* in common carp  
1169 (*Cyprinus carpio*) embryos for transgenic sterilization and reduced sexual maturity.  
1170 Aquaculture 2014;S72–S84:420–21. <https://doi.org/10.1016/j.aquaculture.2013.07.008>.
- 1171 [66] Su B, Shang M, Grewe PM, Patil JG, Peatman E, Perera DA, et al. Suppression and  
1172 restoration of primordial germ cell marker gene expression in channel catfish, *Ictalurus*  
1173 *punctatus*, using knockdown constructs regulated by copper transport protein gene  
1174 promoters: Potential for reversible transgenic sterilization. Theriogenology  
1175 2015;84:1499–512. <https://doi.org/10.1016/j.theriogenology.2015.07.037>.
- 1176