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Article

Generation of Eco-Friendly and Disease-Resistant Channel Catfish (*Ictalurus punctatus*) Harboring the Alligator Cathelicidin Gene via CRISPR/Cas9 Engineering

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ABSTRACT

As a precise and versatile tool for genome manipulation, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) platform holds promise for modifying fish traits of interest. With the aim of reducing transgene introgression and controlling reproduction, upscaled disease resistance and reproductive intervention in catfish species have been studied to lower the potential environmental risks of the introgression of escapees as transgenic animals. Taking advantage of the CRISPR/Cas9-mediated system, we succeeded in integrating the cathelicidin gene (*As-Cath*) from an alligator (*Alligator sinensis*) 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 events 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). The on-target KI of *As-Cath* was instrumental in establishing the *lh* knockout (LH^{-}_As-$Cath^{+}$) catfish line, which displayed heightened disease resistance and reduced fecundity compared with the wild-type (WT) sibling fish. Furthermore, administration of human chorionic gonadotropin (HCG) and luteinizing hormone-releasing hormone analogue (LHRHa) can restore the reproduction of the 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 consumer-valued traits but also guards against unwanted introgression, providing a breakthrough in aquaculture genetics to confine fish reproduction and prevent the establishment of transgenic or domestic genotypes in the natural environment.

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1. Introduction

As science advances, innovative biotechnologies continuously enhance food production, quality, and animal and human welfare. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), a prototype in genome engineering, has unlocked new possibilities in transgenesis and breeding. It operates by triggering two DNA repair mechanisms—non-homologous end joining (NHEJ) and homology-directed repair (HDR)—when it induces double-strand breaks [1]. Both mechanisms have been employed in aquaculture to improve consumer-valued traits within genetic breeding programs. NHEJ is used to knock out (KO) or disrupt functional genes, while HDR is utilized to precisely knock in (KI) exogenous genes of interest, thereby improving target traits.

Recently, various CRISPR/Cas9 systems have emerged to improve the target-editing efficiency for KI via the HDR pathway. Successful applications have been observed in model animals using single-stranded oligodeoxynucleotides (ssODNs) as repair templates, which enables the insertion of small DNA fragments [2–4]. Yoshimi et al. [5] extended the ssODN-mediated approach to KI larger sequences with two 80-bp ssODNs combined with

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CRISPR/Cas9, achieving an approximately 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 obtained [6]. However, erroneous repair was found to be more likely to occur when ssODNs were used as repair templates in zebrafish (*Danio rerio*) [7]. Alternatively, modified donor plasmids with homologous arms (HAs) flanked by two single guide (sgRNA) targeted sequences offer high site-specific KI rates [8,9], and this HA-mediated KI has been adapted to zebrafish and medaka (*Oryzias latipes*) [9,10]. Theoretically, directly offering linear double-stranded DNA (dsDNA) flanked by two HAs derived from the 5'- and 3'- ends of the targeted site while ignoring the difference in stability between circular DNA and dsDNA donors will cause the KI efficiency to increase. In addition to the type of donor, the use of an appropriate concentration of each component of the CRISPR/Cas9 system has a strong positive impact on KI by reducing off-target events and embryo lethality. In this regard, choosing the right delivery system and component dosages holds promise for achieving highly efficient KIs in non-model fish.

Transgenesis and CRISPR/Cas9-mediated genome editing have transformed aquaculture breeding, yielding commercial products such as transgenic AquAdvantage salmon [11,12], gene-edited tiger puffer fish, and red sea bream [13]. While NHEJ dominates for consumer-focused traits such as growth, coloration, and reproduction, HDR-based KIs are effective for improving omega-3 fatty acid content and disease resistance [14–16]. However, the integration of foreign genes via the HDR pathway raises concerns about low KI efficiency and introgression, the latter of which directly impact consumer acceptance of gene-inserted fish [17]. To address this issue, strategies have been developed to make genetically modified fish reproductively sterile using NHEJ-mediated gene disruption, thereby reducing the environmental impacts [17–19]. For example, the luteinizing hormone (LH or *lh*) gene regulates gametogenesis and gestation by 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 (*Ictalurus punctatus*) and white-edged rockfish (*Sebastes taczanowskii*) can result in the production of sterile lines [21,23].

Large-scale disease outbreaks are inevitable, necessitating improved disease-control methods. Antimicrobial peptides (AMPs) are polypeptides that serve as substitutes for antibiotics in a variety of species' initial line of defense against microbial invasions with reduced antibiotic residues [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 [14,26]. Cathelicidins are a particularly important AMP family that share a common cathelin-like domain [27] and exhibit 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 AMGs 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, an additional goal would be to prevent the possibility of the breeding of escapees with wild populations. Hypothetically, a reproductive gene such as *lh* that is 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. The gene-edited sterilized fish from this approach could have their fertility temporarily restored with hormone therapy used for artificial fish spawning, making it 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 *Alligator sinensis* cathelicidin (*As-Cath*) gene at the channel catfish *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 P_1 founders through hormone therapy. In addition, the bacterial resistance of P_1 and their offspring F_1 individuals from the new fish line was further evaluated.

2. Materials and methods

2.1. Ethical approval

The care and use of animals followed the applicable guidelines from expert training courses. Experimental protocols in the current study were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC; Protocol Review Number: 2021-4003). All fish studies were conducted in compliance with the procedures and standards established by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

2.2. Target locus for gene insertion

As the target integration site, we selected the *lh* gene, which is widely expressed in the theca cells of the ovary and aids in egg maturation and ovulation during gonadal development [22]. Based on the published genome of channel catfish [32], the chosen *lh* site for sgRNA targeting is located in the middle of exon 2 (Figs. 1(a) and (b)). The inserted segment is derived from the coding sequence (CDS) of the cathelicidin gene of *Alligator sinensis* (*As-Cath*, GeneBank accession number XM_006037211.3) [29].

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

Gene-targeted KI can be engineered via HDR using dsDNAs or ssODNs as donor templates. In the current study, we employed two CRISPR/Cas9-mediated systems to conduct targeted KI of the *As-Cath* fragment at the *lh* locus. For the first system, the CDS of the *As-Cath* gene was cloned into the pUC57_mini vector at the *EcoRV* enzyme digestion site to create the ssODN1_*As-Cath*_ssODN2 construct as a plasmid donor. Two sgRNAs (sgRNA1 and sgRNA2) were co-injected to operate as "scissors", cutting the *lh* gene and linearizing the plasmid donor, respectively. Two short ssODNs were provided to ligate the ends of both cut sites, labeled as the two-hit two-oligo (2H2OP) system (Fig. 1(a)). ssODN1 consists of 80 bp, of which the upstream 40 bp are derived from the part of exon 2 of the *lh* gene, and the remaining 40 bp are homologous to the pUC57_mini backbone. For ssODN2, the upstream 40 bp are from the pUC57_mini backbone, while the downstream 40 bps come from a portion of exon 2 of the *lh* gene. The dsDNA donor was created by constructing the *As-Cath* CDS sequence flanked with two HAs of 300 bp derived from the *lh* gene of channel catfish on either side of the insert DNA; we tagged the second construct as HA1_*As-Cath*_HA2. More specifically, 163 bp of HA1 (the left homology arm) were 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 the downstream of exon 2, 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; 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

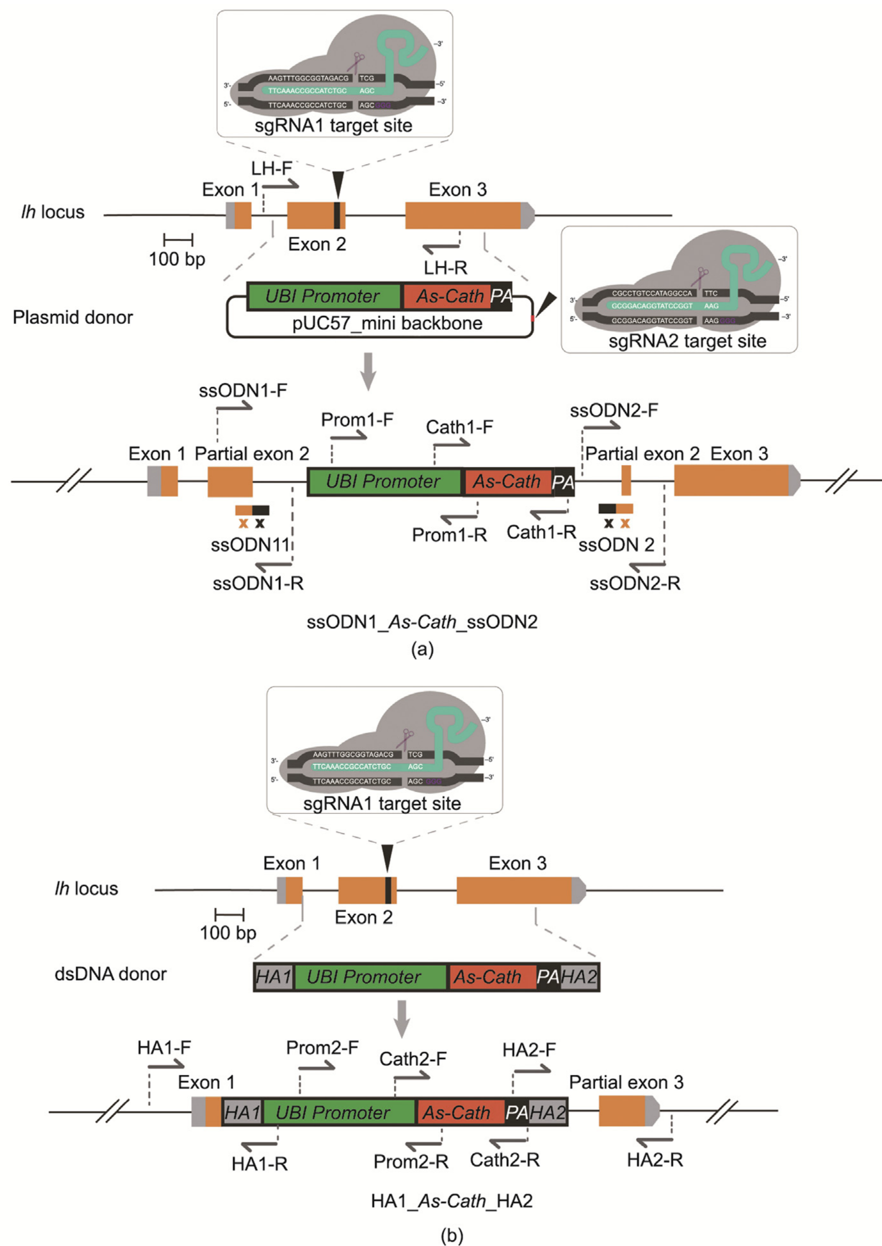

Fig. 1. Design of two CRISPR/Cas9-mediated systems to KI the *Alligator sinensis* cathelicidin (*As-Cath*) transgene at the *lh* locus in channel catfish. (a) Schematic illustration of the insert-specific region for the *As-Cath* KI via the 2H2OP system assisted by ssODNs at the *lh* locus, denoted as the ssODN1_*As-Cath*_ssODN2 construct. The structure of the *lh* gene's exons is constructed by yellow bars, sgRNAs-targeted sites are indicated by black triangles, and the target sequences are detailed in rectangular boxes. The protospacer-adjacent motif (PAM) is highlighted in green. Primer sets are illustrated, showing the strategy to test *lh* mutation, ssODN1/ssODN2 junctions, the UBI promoter region, and the insert-specific region of the *As-Cath* transgene using PCR amplifications. (b) Schematic diagram of the *As-Cath* KI via the dsDNA system, denoted as the HA1_*As-Cath*_HA2 donor. Primers show the strategy to test the HA junctions, the UBI promoter region, and the *As-Cath* transgene region. (HA: homologous arm; UBI: ubiquitin; PA: poly(A) tail.

[33]. The linear dsDNA, circular plasmid, and ssODNs were synthesized by Genewiz (Azenta Life Sciences, USA).

The sgRNAs were selected via the CRISPR design online tool (CRISPR Guide RNA Design Tool, Benching[†]), targeting 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[‡] [34]. Eventually, sgRNA1 for the *lh* locus and sgRNA2 for the donor plasmid were obtained. The Maxiscript T7 kit (Thermo Fisher Scientific, USA) was used to generate sgRNAs *in vitro*, according to the instructions. Next, purified sgRNAs were prepared using the RNA Clean and Concentrator Kit (Zymo Research, USA). The concentration and quality of the sgRNAs were detected with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and 2% agarose gel with 1× tris-borate-EDTA (TBE) buffer, respectively. The synthetic sgRNAs were diluted to a concentration of about 300 ng·μL^{-1} and then divided into polymerase chain reaction (PCR) tubes (2 μL per tube) and stored at −80 °C until use. The Cas9 protein powder was purchased from PNA BIO Inc. (USA); it was then diluted with DNase/RNase-free water to 50 ng·μL^{-1} and kept at −20 °C until use. The sgRNA and universal primer used in this

† https://zlab.bio/guide-design-resources.
‡ http://www.rgenome.net/cas-offinder/.

study are listed in Table S1 (Appendix A). Two different dosages of the donor DNA template and two control groups were set up—50 ng·μL^{-1}, 100 ng·μL^{-1}, sham-injected control (iCT, only the 10% phenol red solution was injected), and non-injected control (nCT, no injection)—for each KI system.

2.4. Transgenic fish production and rearing

Mature channel catfish females and males were collected for artificial spawning according to the guidelines provided by Elaswad et al. [35] with some modifications. In brief, we selected individuals weighing more than 1.5 kg for spawning. Females were anesthetized with 150–200 ppm (1 ppm = 1 mg·L^{-1}) tricaine methanesulfonate (MS222; Hardover, USA) and implanted with 75 μg·kg^{-1} of luteinizing hormone-releasing hormone analog (LHRHa) to induce ovulation. The eggs were then gently stripped in a 20-cm greased spawning pan. Mature males were euthanized with 300 ppm MS222, and then the testes were collected, rinsed, weighed, and crushed; sperm were prepared in 0.9% saline solution (g:v = 1:10). Two milliliters of sperm solution was added to approximately 300 eggs and gently mixed. After 1 min of mixing, sufficient pond water was added to the eggs to activate the sperm, then the sperm/egg mixture was gently swirled for 30 s. More water was added, and the embryos were kept in a single layer in the pan; the embryos were then allowed to harden for 15 min before microinjection.

The CRISPR/Cas9 system used for KI microinjections was combined with Cas9 protein, sgRNA, and donor template in a ratio of 2:1:1, including one component of phenol red as an indicator. For the ssODN1_As-Cath_ssODN2 construct (the 2H2OP system), 8 μL of Cas9 protein (50 ng·μL^{-1}), 2 μL of sgRNA1/sgRNA2 (300 ng·μL^{-1}), 2 μL of donor plasmid (50 and 100 ng·μL^{-1}), 2 μL of ssODN1/ssODN2 (50 and 100 ng·μL^{-1}), and 2 μL of phenol red solution were mixed for microinjection. With respect to the HA1_As-Cath_HA2 construct (the dsDNA system), 4 μL of Cas9 protein (50 ng·μL^{-1}), 2 μL of sgRNA1 (300 ng·μL^{-1}), 2 μL of donor dsDNA (50 and 100 ng·μL^{-1}), 2 μL of phenol red, and 10 μL of DNase-free water were mixed to bring the volume to 20 μL in total. For each mixture of the CRISPR/Cas9 system, we mixed Cas9 protein and sgRNA first and incubated them on ice for 10 min; the donor templates were then supplemented. For the iCT group, we only injected phenol red (diluted with 0.9% saline). The mixed solution for each treatment was microinjected into one-cell stage embryos, as previously described [36]. Every 6 μL of the mixture was loaded into a 1.0-mm outside diameter of borosilicate glass capillary that was pulled into a needle by means of a vertical needle puller (David Kopf Instruments, USA) and injected into 600 embryos. We injected 1000 embryos, dividing them into five random replicates for each treatment; another 200 embryos with three replicates were prepared for each control group. All 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 of Holtfreter solution (59 mmol NaCl, 2.4 mmol $NaHCO_3$, 1.67 mmol $MgSO_4$, 0.76 mmol $CaCl_2$, and 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; the dissolved oxygen levels were maintained at >5 ppm via continuous aeration. Holtfreter solution was replaced twice per day, and dead embryos/fry were collected and recorded daily during hatching for analysis. The hatched fry were transferred to a Holtfreter solution without doxycycline and fed with live *Artemia* nauplii four times per day. After 1 week of culture in tubs, all fry were stocked separately into a 60-L aquaria (120 fish per tank) in a recirculating system for growth. The feed pellet size was adjusted according to the size of the fish's mouth as the fish grew. In detail, the fry in tanks were fed with Purina AquaMax powdered feed (50% crude protein, 17% crude fat, 3% crude fiber, and 12% ash) four times per day for 2 months. Then, the 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 2 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 [15]. 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., USA) to distinguish each individual. The fish from different treatments were then mixed together and randomly dispersed into two circular tanks (1200-L volume filled with ~800 L of water) with the same density (120 fish per tank) for monthly growth comparison. 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. For the ssODN1_*As-Cath*_ssODN2 construct, the CDS region of *As-Cath* was amplified to confirm gene insertion using the primers Cath1-F/R (forward and reverse), and the promoter region was amplified via the primers Prom1-F/R. As for the junctions, the ssODN1 and ssODN2 regions were amplified using the primers ssODN1-F/R and ssODN2-F/R to determine whether it was an on-target insertion. For the HA1_*As-Cath*_HA2 construct, the *As-Cath* and promoter regions were detected using the primers Cath2-F/R and Prom2-F/R, respectively. Then, the left HA and right HA junctions were amplified via the primers HA1-F/R and HA2-F/R. The primers were designed using the online software Primer3Plus[§] and are listed in Table S2 (Appendix A). PCR was performed in a 10-μL system, and the PCR products were visualized by running 1.0% agarose gel with 1× tris-acetate-EDTA (TAE) buffer. A bright band of each region with the corresponding length indicated an on-target positive (LH^-_As-$Cath^+$). Here, if we could determine that some individuals had been inserted with the *As-Cath* transgene but could not detect the junctional regions (HA- or ssODN-region), we identified them as potential off-target positives (LH^+_As-$Cath^+$).

With respect to the LH^+_As-$Cath^+$ fish, we selected 60 individuals to be tested for *lh* mutations. In this case, PCR was performed in a 20-μL volume system using an Expand High Fidelity^{PLUS} PCR System (Roche Diagnostics, USA) according to Elaswad et al. [35], and LH-F/R primers were used in both constructs. Next, a surveyor mutation detection assay was performed via a Surveyor Mutation Detection Kit (IDT, 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 h in 2% agarose gel using 1× TBE buffer and compared with wild-type (WT) samples.

2.6. DNA sequencing

For the integrated *As-Cath*, promoter, and junction sequences, PCR of the positive samples was performed in a 50-μL volume system. The PCR products were then purified using a QIAquick PCR Product Purification Kit (QIAGEN, Germany) according to the manufacturer's instructions. Before sequencing, all purified DNA samples were quantitated and identified using Nanodrop and by running electrophoresis using 1.0% agarose gel. The primers

§ http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi.

Cath1-F/Cath2-F and Prom1-F/Prom-2F were used for the sequencing of *As-Cath* and promoter regions for HA1_*As-Cath*_HA2 and ssODN1_*As-Cath*_ssODN2 constructs, respectively; the primers HA1-F/HA2-F and ssODN1-F/ssODN2-F were used for the sequencing of junctional regions for these two constructs, respectively.

Regarding *lh* mutations, we cloned the PCR products of putative mutant individuals using a TOPO TA Cloning Kit (Invitrogen, USA), following the instructions with some modifications. In brief, PCR was performed on each mutant individual that was previously identified with a Surveyor assay using the primers LH-F/R for the next cloning steps. In addition, the DNA of three WT individuals from the nCT group was prepared using the same primers and procedures, and then combined into one reaction and cloned as a WT control for sequencing. After cloning, we transformed the pCR™4-TOPO vector containing the PCR products into One Shot TOP10 Electrocomp™ *Escherichia coli* (*E. coli*) (Invitrogen, USA), 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.

2.7. Determination of mosaicism and transgene expression

Five 12-month-old on-target positive fish and five sham-injected control fish were randomly chosen and sacrificed. Fourteen tissues, including the 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. The total RNAs were isolated from various tissues using TRIzol reagent (Thermo Fisher Scientific, USA) and were reverse-transcribed to cDNA using an iScript™ Synthesis Kit (Bio-Rad, USA), following the manufacturer's protocols.

qRT-PCR was performed on a C1000 Thermal Cycler using a SsoFast™ EvaGreen Supermix kit (Bio-Rad, USA), according to the instructions. Concentrations of the cDNA products were diluted to 250 $ng\cdot\mu L^{-1}$, and a 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 S2. CFX Manager Software (version 1.6, Bio-Rad) was used to collect the raw crossing-point (C_t) 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_1 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_1 individuals are expected to reach sexual maturity [40]. To evaluate the reproduction of 2-year-old KI founders, on-target positive (LH^-_As-$Cath^+$), off-target positive (LH^+_As-$Cath^+$), and WT fish were selected to conduct a three-round mating experiment. Firstly, three pairs of WT, six pairs of LH^-_As-$Cath^+$, and four pairs of LH^+_As-$Cath^+$ mature parents were randomly placed into 13 tanks (60 cm × 45 cm × 30 cm) for two weeks of natural spawning to evaluate the spawnability of each genotype, and egg masses were collected from the spawnable parents. We then primed the males with a 50 $\mu g\cdot kg^{-1}$ LHRHa implant and 1600 $IU\cdot kg^{-1}$ human chorionic gonadotropin (HCG) in the unspawned groups with a 1-week observation period to determine whether the LH^-_As-$Cath^+$ females were fertile. After this period, we recruited six more pairs of LH^-_As-$Cath^+$ fish to assess the effects of hormone therapy using three dosages of a combination of HCG and LHRHa implant (1200 $IU\cdot kg^{-1}$ HCG + 50 $\mu g\cdot kg^{-1}$ LHRHa, 1600 $IU\cdot kg^{-1}$ HCG + 50 $\mu g\cdot kg^{-1}$ LHRHa, 2000 $IU\cdot kg^{-1}$ HCG + 50 $\mu g\cdot kg^{-1}$ LHRHa) and a control group injected with 0.85% NaCl. A 30-g egg mass for each genotype, with three replicates, was collected to calculate the fecundity ($eggs\cdot kg^{-1}$ 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_1 fish

All the fry were separated into 60-L tanks by different genotypes. After four months of culture, fin clips and barbels were collected for DNA extraction from 60 F_1 individuals of each genotype, except the control groups. The same culture and genotyping procedures as described above were applied to the F_1 generation.

2.10. Experimental challenge with Flavobacterium covae (F. covae) and Edwardsiella ictaluri (E. ictaluri)

Gene-edited channel catfish were cultured in 60-L aquariums in the greenhouse of the Fish Genetics Laboratory at Auburn University (approved by AU-IACUC). To determine the resistance against pathogens, both P_1 and F_1 fish were challenged by *F. covae* and *E. ictaluri.*

2.10.1. F. covae challenge

Healthy P_1 fingerlings with a BW of (150.62 ± 4.24) g (mean ± standard error), including four genotypes (15 fish per genotype)—that is, LH^-_As-$Cath^+$, LH^+_As-$Cath^+$, negative LH^+_As-$Cath^-$ (negative fish without *As-Cath* insertion and *lh* mutation), and WT—were mixed and acclimated in one hatching trough for five days and then transferred to a 1800-L tank in the challenge room for acclimation for another 24 h prior to bacterial infections. All fish were randomly/equally separated into two 60-L buckets (30 L of water). In brief, a revived *F. covae* isolate (strain ALG-00-530) on modified Shieh agar (MSA) was inoculated into multiple cultures of 12 mL of modified Shieh broth (MSB) in 50-mL sterile flasks and grown in a shaker incubator at 150 $r\cdot min^{-1}$ for 12 h at 28 °C. These cultures were then expanded into 200-mL cultures (5-mL additions) in 500-mL flasks and grown for another 12 h. The optical density (OD) was adjusted to OD_{540} = 0.731; then, spread plate dilutions were performed to determine the final inoculum concentration. One hundred microliters of each inoculum was serially diluted and spread onto MSA agar plates in duplicate and incubated at 28 °C for 48 h to quantify the concentration of the inoculum. Two flasks containing 325 mL of inocula (4.55×10^8 $CFU\cdot mL^{-1}$) were immediately added to two 60-L buckets with fish following preparation, respectively. The fish were then immersed statically in buckets for 1.5 h at approximately 28 °C (immersion dose: 2.46×10^6 $CFU\cdot mL^{-1}$); afterward, all fish were gently moved back into the 1800-L tank containing 1000 L of water, and water flow was resumed. Meanwhile, a mock-challenged tank was used as the control, incorporating another 40 fish in 30 L of rearing water for 1.5 h with sterile MSB (325 mL) instead of the bacterial culture. With respect to the challenge of the F_1 fry ((3.15 ± 0.24) g), four families of F_1 fry (45 fish per family)—namely, LH^-_As-$Cath^+$, LH^+_As-$Cath^+$, LH^+_As-$Cath^-$, 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 4.75×10^8 $CFU\cdot mL^{-1}$ (immersion dose: 2.57×10^6 $CFU\cdot mL^{-1}$) were implanted for the F_1 generation.

2.10.2. E. ictaluri *challenge*

Sixty P_1 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, USA. The detailed procedures of the *E. ictaluri* challenge were performed according to Simora et al. [30], with some modifications. In brief, 1 mL of frozen glycerol stock of *E. ictaluri* was inoculated into 20 mL of brain–heart infusion broth (BHIB; Hardy Diagnostics) at 26 °C in a shaker incubator at 180 $r \cdot min^{-1}$ for 24 h. Bacteria were then subcultured into 1 L of BHIB for another 24 h under the same conditions until the cell density reached about 1×10^8 $CFU \cdot mL^{-1}$, based on the OD_{600} value. All P_1 individuals were transferred into one 1800-L tank for the challenge. Before starting *E. ictaluri* infection, the water was lowered to a total of 100 L; then, 1 L of *E. ictaluri* suspension containing 3.20×10^8 $CFU \cdot mL^{-1}$ cells was added to the tank, resulting in a final immersion dose of 3.20×10^6 $CFU \cdot mL^{-1}$. The fish were immersed statically for 2 h with aeration > 5 ppm, and then the water was restored. In addition to the infected groups, one control tank containing 30 fish received only BHIB as a mock-challenged group. With respect to the challenge of the F_1 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 2.80×10^8 $CFU \cdot mL^{-1}$ (immersion dose: 2.80×10^6 $CFU \cdot mL^{-1}$), were implanted for the F_1 generation.

During the first 72 h of the experiment, we checked for mortality every 4 h and then three times daily. Challenged fish were continuously monitored for ten days for external clinical signs of *F. covae*/*E. ictaluri* and confirmation of bacteria colony growth was obtained by isolating bacteria from the kidney and liver to determine the cause of death. Dead individuals were recorded and collected over time.

2.11. Statistical analysis

Spawnability, hatchability, fecundity, fry survival rate, and growth data were analyzed using one-way analysis of variance (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 A) were adopted. The survival curves of the challenge experiments were determined using Kaplan-Meier plots followed by a 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 ± standard error of the mean (SEM).

3. Results

3.1. Targeted KI of As-Cath gene into the lh locus

Both the 2H2OP and dsDNA systems were able to induce *As-Cath*-integrated catfish lines with high integrated rates, but the 2H2OP system had significant off-target effects (Figs. 2(a) and (b), Figs. S1–S4 in Appendix A). More specifically, the 2H2OP system containing 50 $ng \cdot \mu L^{-1}$ 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 S4 in Appendix B). Although the 2H2OP50 group was able to introduce the highest KI efficiency ($P < 0.01$) (Fig. 3(a)), the 2H2OP system or 50 $ng \cdot \mu L^{-1}$ of donors brought a significantly higher KI efficiency than the dsDNA method ($P = 0.0001$) or 100 $ng \cdot \mu L^{-1}$ of donors ($P = 0.00469$) (Figs. 3(b) and (c)). However, the dsDNA with 50 $ng \cdot \mu L^{-1}$ donors demonstrated the highest on-target KI efficiency (10.80%, 23/213) compared with the other treatments ($P < 0.01$) (Fig. 3(d)). In contrast, only one on-target KI case was observed in the 2H2OP system, which was significantly lower than in the dsDNA ($P < 0.0001$) (Fig. 3(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. 3(f)).

According to the OR, the 2H2OP system and a low dosage tended to bear a higher total integrated rate, which was 2.30 and 1.47 times greater than that of the dsDNA (OR = 2.30 for 2H2OP vs dsDNA) and high dosage (OR = 1.47 for 50 vs 100 $ng \cdot \mu L^{-1}$), respectively. Nonetheless, dsDNA showed an overwhelming superiority in on-target integration, which was more than 20 times greater than that in the 2H2OP system (OR = 26.70) (Table S3). Taken together, the findings showed that the dsDNA system accompanied by a dosage of 50 $ng \cdot \mu L^{-1}$of donors yielded the highest on-target KI efficiency in our current study.

Given the non-*As-Cath*-integrated fish, we did detect individuals with only the *lh* mutation. More specifically, 5.56% (3/54), 6.67% (4/60), 3.33% (2/60), and 3.33% (2/60) of fish with *lh* deficiency in the 2H2OP50, 2H2OP100, dsDNA50, and dsDNA100 groups, respectively, were detected by the Surveyor mutation test (Table S4 in Appendix A). The sequencing results revealed 2, 2, 1, and 3 types of mutations in 4 *lh*-mutant individuals from the 2H2OP100 group (Fig. S5 in Appendix A).

3.2. Effects of the dosage and CRISPR/Cas9 system

Different donor dosages and CRISPR/Cas9-mediated systems exhibited toxicity to the fish embryos, decreasing the hatchability and fry survival rate. Although there were no significant differences in hatching rates among these four CRISPR/Cas9-mediated injected groups compared with the iCT group ($P = 0.1630$), the hatching rate was lower than that of the nCT group ($P < 0.01$) (Fig. 3(g)). Moreover, the lethality of embryos was consistent across different donor dosages (50 vs 100 $ng \cdot \mu L^{-1}$) ($P = 0.1080$) or CRISPR/Cas9-mediated systems (2H2OP vs dsDNA) ($P = 0.0796$), and was significantly higher than that in the nCT group (Figs. 3(h) and (i)). Regarding fry survival, high survival rates of over 90% were observed in all groups ($P = 0.0747$; Fig. 3(j)) without significant effects from the CRISPR/Cas9 system (2H2OP vs dsDNA, $P = 0.9975$; Fig. 3(k)) and dosages (50 vs 100 $ng \cdot \mu L^{-1}$, $P = 0.9995$; Fig. 3(l)).

3.3. Mosaicism and As-Cath expression

PCR and qRT-PCR were used to detect the *As-Cath* transgene and its expression in the different tissues of on-target positive fish. The results revealed that three of the five LH^-_$As\text{-}Cath^+$ fish expressed the *As-Cath* in all 14 sampled tissues (skin, liver, kidney, spleen, blood, intestine, gill, stomach, fin, barbel, muscle, eye, brain, and gonad) (Figs. 4(a) and (b)), but one of them had expression in 11 tissues (except barbel, muscle, and gill) and another in eight tissues (skin, liver, blood, intestine, gill, barbel, muscle, and gonad) (Fig. S6 in Appendix A), suggesting mosaicism in the on-target positive individuals. We found that the expression of *As-Cath* was detected even without pathogenic infections for the three on-target positive individuals. The three highest mRNA levels were determined in the kidney (28.91-fold changed), skin (24.30-fold), and gill (8.45-fold), followed by the muscle (7.43-fold), spleen (6.05-fold), and barbel (4.81-fold). However, the eye (1.33-fold), intestine (1.59-fold), and fin (1.61-fold) had the lowest expression compared with other tissues (Fig. 4(c)).

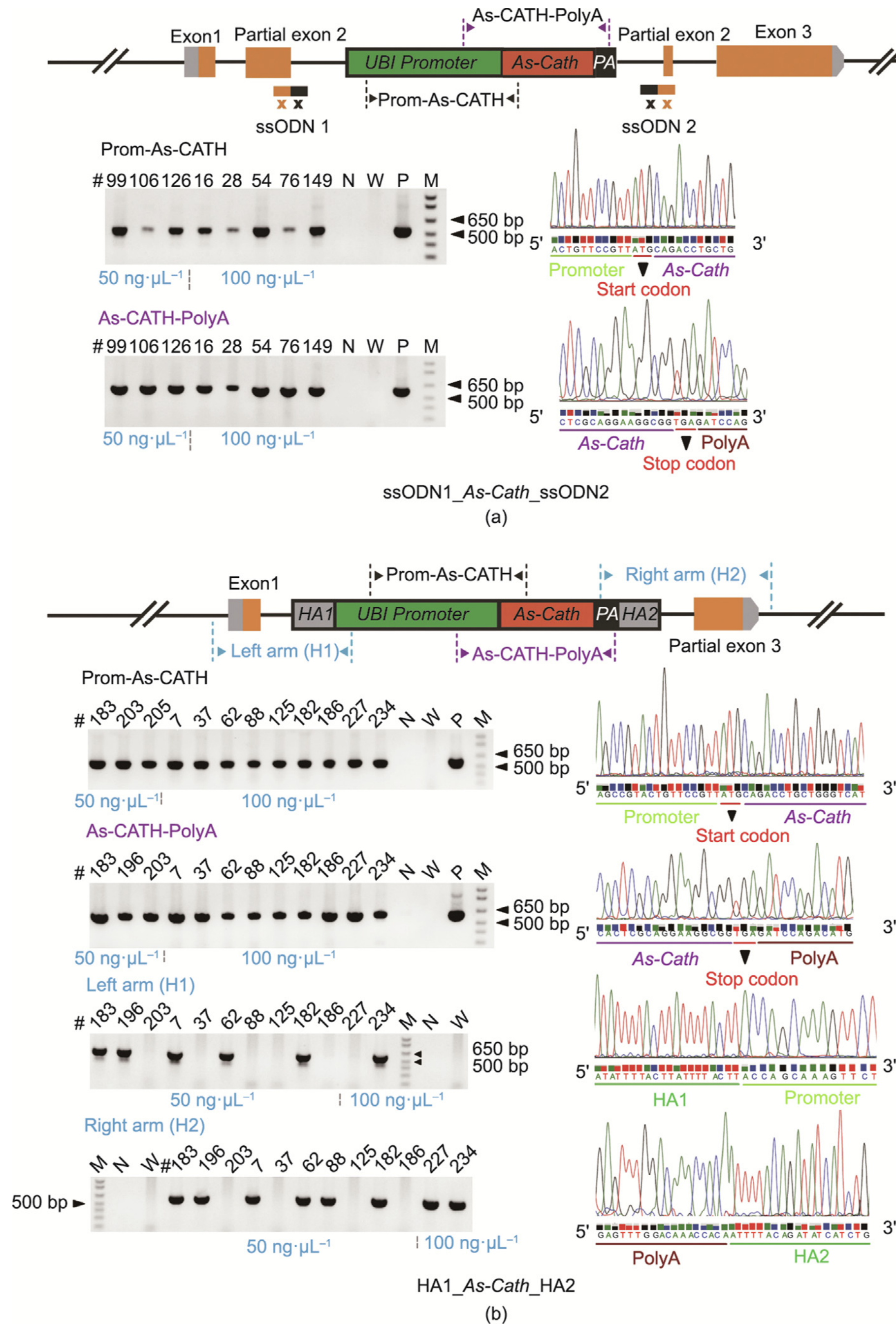

Fig. 2. Detection of transgenic positive channel catfish carrying the *As-Cath* transgene. (a) TAE agarose gel of PCR amplicons showing off-target positive detection of the ssODN1_*As-Cath*_ssODN2 construct using the 2H2OP method. The promoter region (Prom-As-CATH, 519 bp) and *As-Cath* region (As-CATH-PolyA, 591 bp) are illustrated with the sequencing results. (b) TAE agarose gel of PCR amplicons showing the on-target positive detection of the HA1_*As-Cath*_HA2 construct using the 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^{-1} show the different doses of donors: plasmid or dsDNA.

In addition, compared with the WT individuals, the mRNA level of *lh* in gonads was down-regulated in LH$^-$_As-Cath$^+$ females at the age of one year ($P = 0.0016$), but there was no significant difference in that of males ($P = 0.5817$) (Fig. 4(d)). LH levels in the body are often regulated by feedback mechanisms involving sex hormones such as estrogen and testosterone. Females typically have higher estrogen levels, which can affect LH secretion. Any disruptions to these hormonal feedback loops may impact LH mRNA levels more prominently in females.

3.4. Reproductive sterility and restoration of reproduction

A three-round mating experiment determined the potential for complete control of channel catfish reproduction (Fig. 5(a)). Our outcomes revealed that three pairs of WT (100%, 7927 eggs·kg^{-1} BW) and two pairs of LH$^+$_As-Cath$^+$ fish (50%, 8952 eggs·kg^{-1} BW) were respectively spawned during the first 2-week natural mating, but no spawn was observed in the LH$^-$_As-Cath$^+$ pairs (0%). Compared with the LH$^-$_As-Cath$^+$ pairs, the WT and LH$^+$_As-

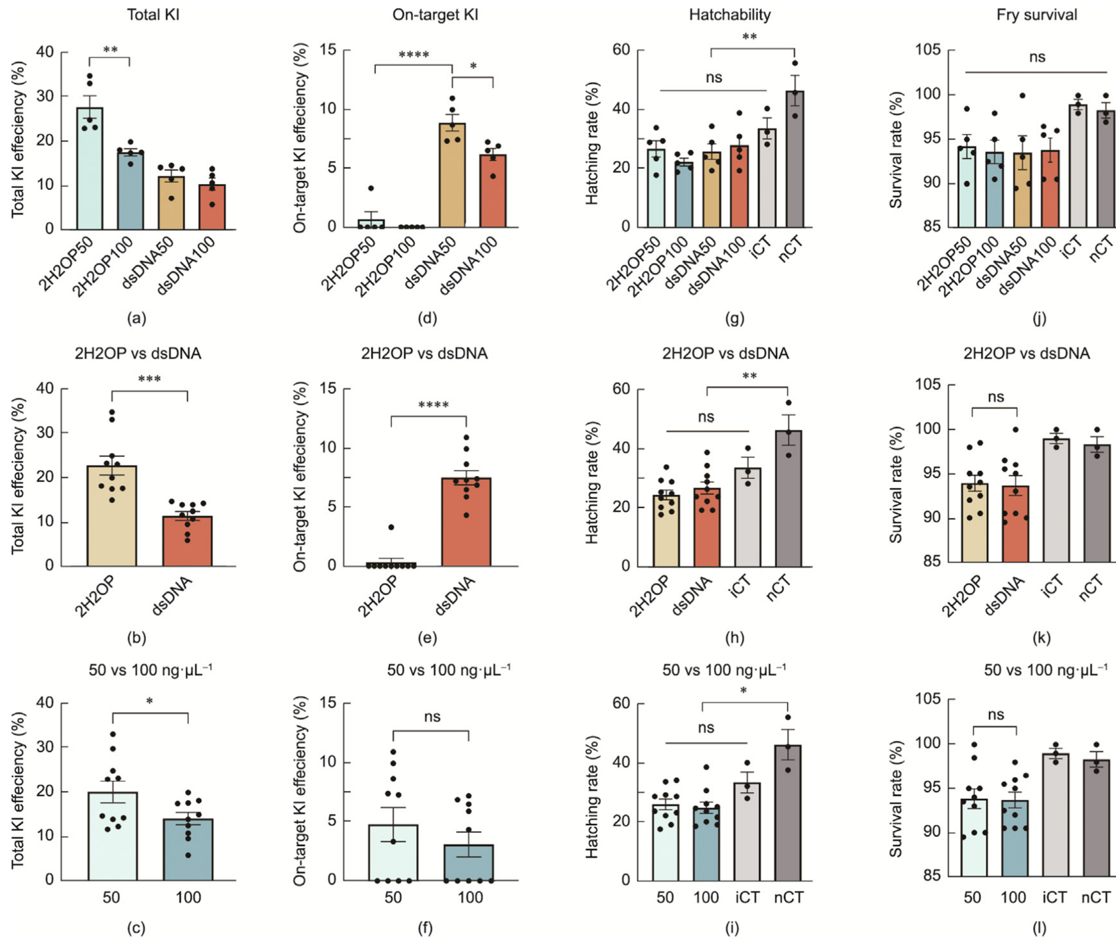

Fig. 3. Effects of different CRISPR/Cas9-mediated systems on the KI efficiency, hatchability, and fry survival rate. (a) Total KI efficiency of different CRISPR/Cas9-mediated systems and dosage combinations. (b, c) Comparison of total KI efficiency for different systems or dosages of donors. (d) On-target KI efficiency of different CRISPR/Cas9-mediated systems and dosage combinations. (e, f) Comparison of the on-target KI efficiency of different systems or dosages. (g) Effect of different CRISPR/Cas9-mediated systems and dosage combinations on hatchability. (h, i) Comparison of the hatchability for different systems or dosages. (j) 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. 2H2OP(50/100): the CRISPR/Cas9-mediated system coupled with the ssODN1_*As-Cath*_ssODN2 construct (with a pUC57_mini plasmid and ssODN donor as 50/100 ng·μL^{-1}); dsDNA(50/100): the CRISPR/Cas9-mediated system coupled with the HA1_*As-Cath*_HA2 donor (with a dsDNA donor as 50/100 ng·μL^{-1}) .* 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.

Cath$^+$ fish had higher spawnability under natural pairing conditions (P = 0.0148 and P = 0.1743). In addition, the LH$^+$_As-Cath$^+$ pairs did not show a significant difference in spawnability compared with the WT pairs (P = 0.2143) (Fig. 5(b)).

Furthermore, the 1-week hormone priming (50 μg·kg^{-1} LHRHa + 1600 IU·kg^{-1} HCG) of the males did not stimulate the LH$^-$_As-Cath$^+$ females to give eggs, indicating that the *lh*-deficient females blocked oocyte maturation and ovulation. However, our results indicated that a combination of LHRHa and HCG effectively induced spawning for the LH$^-$_As-Cath$^+$ females when both males and females were primed. More specifically, two, two, and one female gave eggs 24–48 h post-hormone injection from the 1200 IU (6213 eggs·kg^{-1} BW), 1600 IU (5514 eggs·kg^{-1}g BW), and 2000 IU·kg^{-1} (3778 eggs·kg^{-1} BW) HCG group combined with 50 μg·kg^{-1} LHRHa, respectively. These three treatments significantly improved the fecundity compared with 0.85 % NaCl injection (P < 0.0001). The fecundity decreased with increasing hormone dosage, but the difference among these three hormone dosages was not significant (P = 0.0731). Nevertheless, the fecundity was restored to a normal level when 1200 (P = 0.2627) or 1600 (P = 0.1983) IU·kg^{-1} HCG combined with 50 μg·kg^{-1} LHRHa was adopted (Fig. 5(c)). Compared with the WT and the other hormonal-therapy groups, the 2000 IU·kg^{-1} HCG group significantly reduced the fecundity (3778 eggs·kg^{-1} BW, P = 0.0494) and hatchability (18.01%, P = 0.0476) (Fig. 5(d)). Although different hormone treatments had varying effects on fecundity and hatchability, they had no effects on fry survival at the early stage (P = 0.1018) (Fig. 5(e)).

3.5. F_1 genotyping, growth comparison in P_1 and F_1

As mentioned above, three WT, two LH$^+$_As-Cath$^+$, and five LH$^-$_As-Cath$^+$ families were generated from our three-round mating experiment. However, genotype analysis determined that only one family of the LH$^+$_As-Cath$^+$ line (33.33% (10/30) integrated rate in the F_1 offspring) and two families of the LH$^-$_As-Cath$^+$ line (40% (12/30) integrated rate in the F_1 progeny of family 1 and 46.67% (14/30) integrated rate in the F_1 offspring of family 2) had the *As-Cath* gene detectable in the F_1 generation. These results further confirmed the existence of the mosaic phenomenon in the P_1 founders.

To determine the pleiotropic effects of *lh* disruption and *As-Cath* integration on fish growth, we compared the BW of the P_1 founders and the F_1 progeny, respectively, over time. The growth data suggested that the LH$^-$_As-Cath$^+$ individuals did not show superiority in terms of growth in the first nine months in the P_1 generation.

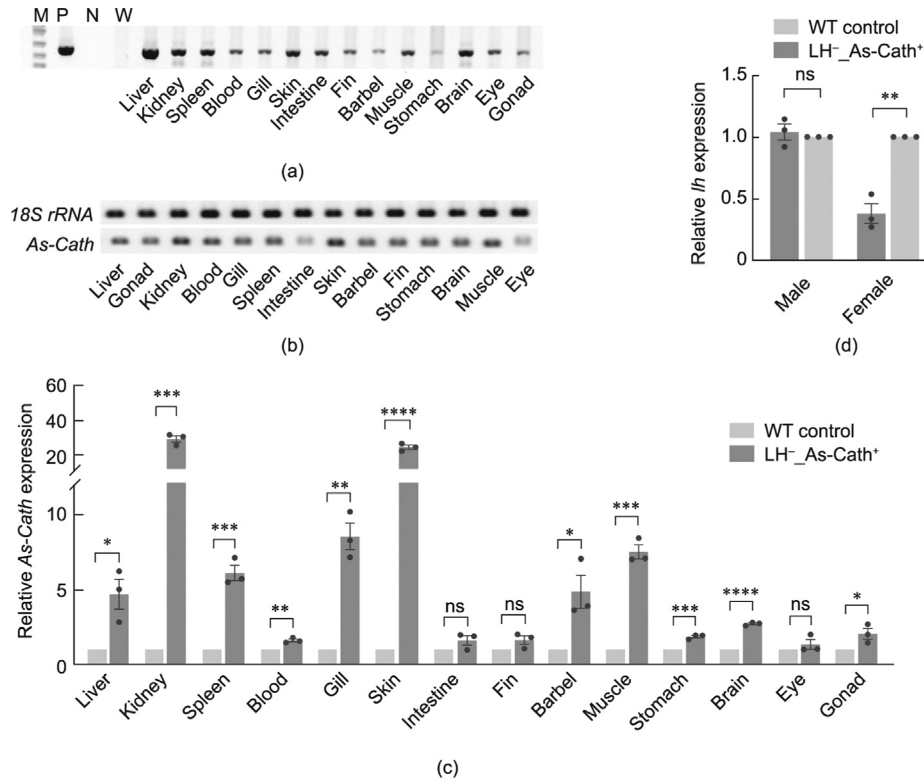

Fig. 4. Mosaicism detection and the expression of the cathelicidin gene from *Alligator sinensis* (*As-Cath*) in the LH$^-$_As-Cath$^+$ channel catfish line. (a) PCR amplicons showed the *As-Cath* transgene in 14 tissues from one representative LH$^-$_As-Cath$^+$ fish. (b) Agarose gel electrophoresis showed the *As-Cath* gene expression in various tissues of P_1 transgenic channel catfish. (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. *18S rRNA* was utilized as an internal control. The gene expression level of control was set as 1.0. Data are shown as average fold change relative to the control (mean ± standard error, n = 5). Expression levels were calibrated against corresponding tissues from sibling WT fish, and five 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).

Nonetheless, P_1 LH$^-$_As-Cath$^+$ fish exhibited the largest body gain (36.35 g) compared with other genotypes (25 g). Furthermore, significantly faster growth (P = 0.0222) was demonstrated in the F_1 generation of LH$^-$_As-Cath$^+$ after a 3-month culture (Table S5).

3.6. Enhanced resistance against fish pathogens

Dead fish were diagnosed and confirmed with bacterial infection (*F. covae* or *E. ictaluri*) based on clinical symptoms and bacterial cultures from their liver and kidneys. Enhanced resistance against bacteria in *As-Cath*-integrated fish was observed compared with WT/negative individuals from our challenge experiments in both P_1 and F_1 generations. According to the *F. covae* challenge observation, most of the infected WT fish showed visible clinical signs: saddleback, fin erosion, and yellow skin discoloration on the body. However, some *As-Cath* transgenic fish showed mild clinical signs, with frayed fins, but survived the infection. There was no significant difference in survival rate between the two types of controls (WT and LH$^+$_As-Cath$^-$) in both P_1 (13.33% vs 20%, P = 0.8682) and F_1 generation (26.67% vs 40%, P = 0.8955). However, the LH$^-$_As-Cath$^+$ and LH$^+$_As-Cath$^+$ fish exhibited significantly improved survival post-*F. covae* infection compared with the WT group in both P_1 founders (LH$^-$_As-Cath$^+$ vs WT: 73.33% vs 13.33%, P = 0.0016; LH$^+$_As-Cath$^+$ vs WT: 66.67% vs 13.33%, P = 0.0014) and F_1 progeny (LH$^-$_As-Cath$^+$ vs WT: 86.67% vs 26.67%, P = 0.0010; LH$^+$_As-Cath$^+$ vs WT: 73.33% vs 26.67%, P = 0.0127). In addition, the on-target insertion of the *As-Cath* gene resulted in improved resistance against *F. covae* in comparison with the off-target positives, without statistically differing in both generations (73.33% vs 66.67%, P = 0.7726 for P_1, and 86.67% vs 73.33%, P = 0.3613 for F_1). Furthermore, our findings revealed that the F_1 progeny was more resistant to *F. covae* than its P_1 parent (Figs. 6(a) and (b)).

The typical "hole-in-the-head" clinical sign was observed in dead WT channel catfish, accompanied by an accumulation of ascites, a hemorrhage, and exophthalmia. However, some *As-Cath* transgenic fish showed skin ulcers and swollen bellies but survived the infection. Increased resistance to *E. ictaluri* was also observed in the P_1 (LH$^-$_As-Cath$^+$ vs WT: 73.33% vs 33.33%, P = 0.0125; LH$^+$_As-Cath$^+$ vs WT: 60% vs 33.33%, P = 0.0427) and F_1 generations (LH$^-$_As-Cath$^+$ vs WT: 66.67% vs 40%, P = 0.0558; LH$^+$_As-Cath$^+$ vs WT: 73.33% vs 40%, P = 0.0350), with results similar to those of the *F. covae* challenge. Overall, *As-Cath*-integrated individuals showed a significant improvement in survival rate compared with WT fish (66.67% vs 33.33%, P = 0.0381 for P_1; 70% vs 40%, P = 0.0335 for F_1). Nevertheless, there was no significant difference in LH$^-$_As-Cath$^+$ and LH$^+$_As-Cath$^+$ fish (73.33% vs 60%, P = 0.4566 for P_1; 66.67% vs 73.33%, P = 0.6851 for F_1) (Figs. 6(c) and (d)).

4. Discussion

In contrast to previous gene-editing oriented exclusively to improving the desired traits, the present study took into account ways to decrease the potential impact of transgenic fish on ecosystems and genetic biodiversity. More specifically, we successfully integrated an AMG into a reproduction-associated locus using CRISPR/Cas9-mediated engineering. We identified a suitable KI system for channel catfish to achieve boosted resistance against

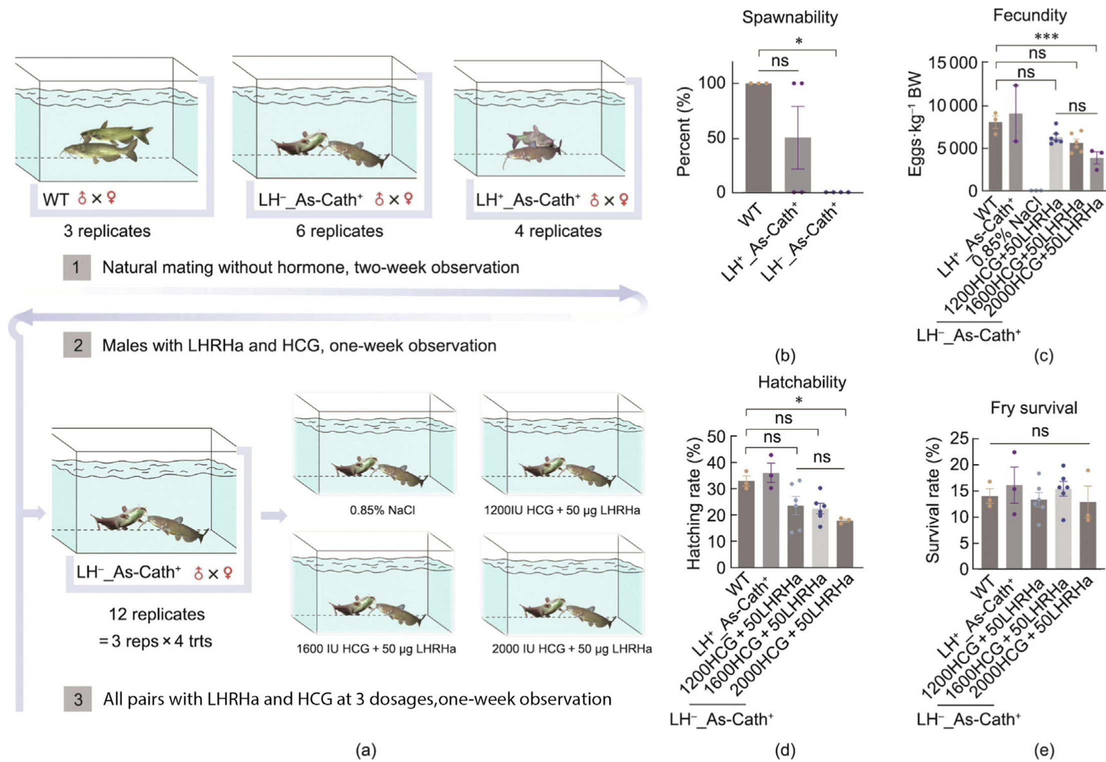

Fig. 5. Reproductive determination and restoration of the *As-Cath*-integrated channel catfish lines. (a) A three-round design of the reproduction experiment. Three genotypes of P_1 founders were involved: WT, LH⁻_As-Cath⁺, and LH⁺_As-Cath⁺ fish. First round: Three, six, and four pairs as replicates for each genotype were set up randomly in 13 tanks for mating without hormone treatments, and a 2-week observation was adopted. Second round: Spawned pairs were moved out and un-mated males were primed with a 50 μg·kg^{-1} LHRHa implant and 1600 IU·kg^{-1} HCG to determine the reproduction of LH⁻_As-Cath⁺ females, observing for one week. Third round: 12 pairs of LH⁻_As-Cath⁺ fish were complemented and re-paired and treated with three dosages of LHRHa and HCG for one week. In total, there were four treatments, with three replicates for each group. (b) Detection of spawnability for LH⁻_As-Cath⁺ fish during natural mating. (c–e) Potential effects of different hormone treatments on the fecundity and hatchability of P_1 generation, and fry survival of F_1 generation. * $P < 0.05$; ** $P < 0.01$; ns = not significant, by unpaired Student's *t*-test or one-way ANOVA.

fish pathogens and reproductive control, with the aim of 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 was found to be entirely controllable and could only be restored with hormone therapy in the new fish line. In general, the insertion of the cathelicidin transgene 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.

Several obstacles appear 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. When moving from model to non-model animals, there are several uncertainties (e.g., embryo size, developmental period, and sensitivity to Cas9 protein) for which researchers must 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 have indicated that ssODN-mediated KI can induce a high percentage (17.8%) of indel mutations in sheep [41]. In the current study, we used CRISPR/Cas9 systems assisted by ssODNs and HAs 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 agree 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 with the ssODN-mediated system, HA-assisted KI can achieve a 20%–30% HDR-mediated KI in human cells with various homogeneous sequences [9,43]. In addition, Simora et al. [44] determined that HA-mediated CRISPR/Cas9 provided with a linear dsDNA donor displayed a total integration rate of 29% at the non-coding region of the 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 than 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, novel CRISPR/Cas-mediated techniques are constantly being developed. For example, the CRISPR/Cas12i-mediated system shows promise in multiplexed genome editing with high mutation rates in human T cells [45]. Moreover, using homology-independent targeted integration (HITI) vectors, Kelly et al. [46] established a CRISPR/Cas9 HITI system for the insertion of large DNA donors, with a high integrated efficiency of 36% in human 293T cells. Recently, a novel study demonstrated that long sequences can be integrated in mammalian cells using the single-strand annealing proteins (SSAPs) with catalytically inactive dCas9 (dCas-SSAP) sys-

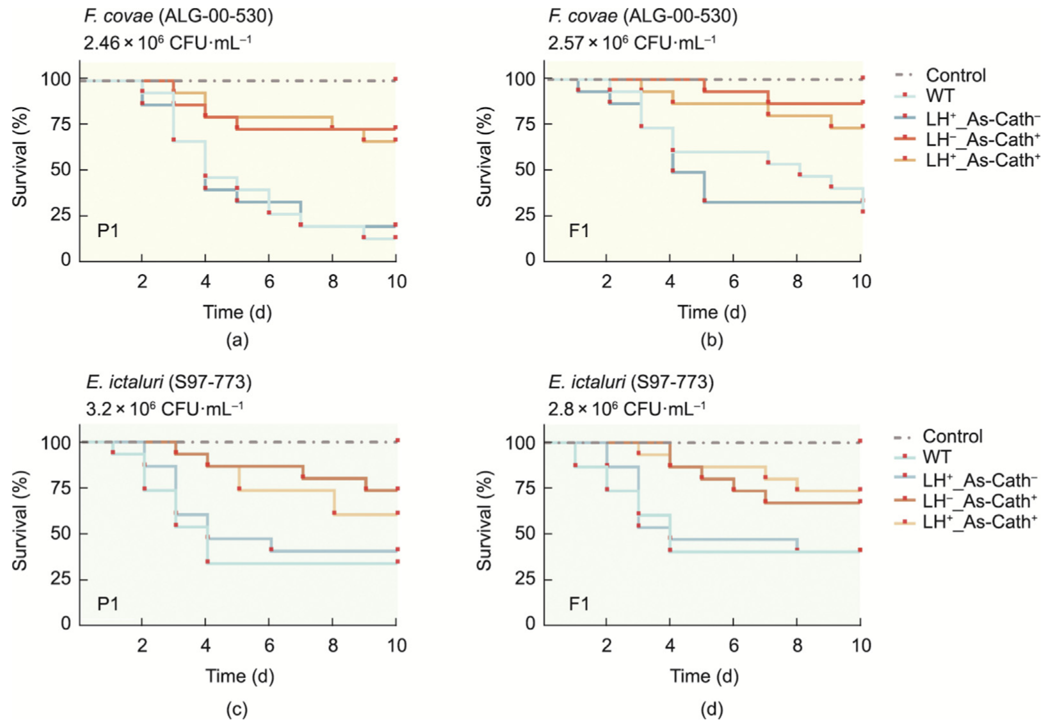

Fig. 6. Kaplan-Meier plots of *As-Cath*-integrated channel catfish against two fish bacterial pathogens. (a, b) Survival curves of P_1 and F_1 generations for a variety of genotypes infected by *F. covae*, respectively. (c, d) Survival curves of P_1 founder and F_1 progeny for different genotypes infected by *E. ictaluri*, respectively. Comparison of different survival curves was determined by the log-rank (Mantel-Cox) test. Non-injected fish line; LH^+_As-$Cath^-$: negative fish line (micro-injected fish without *lh* mutation and *As-Cath* insertion); LH^-_As-$Cath^+$: on-target positive fish (*As-Cath* insertion was detected at *lh* locus); LH^+_As-$Cath^+$: off-target positive fish (*As-Cath* insertion was detected but not at *lh* locus).

tem with a high on-target KI efficiency (~20%) [47]. These new tools or systems hold promise for application from model to non-model animals and could improve genome-editing efficiency.

Although we predicted and avoided possible off-target events using well-acknowledged software, the actual integration results showed the existence of off-target activities. This was 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 animal experiments *in vivo* than in cellular experiments *in vitro* [50]. Most publications contend that the observed unintended mutations/insertions are a major concern in the application of the CRISPR/Cas9 system and 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 potential can be noted: (1) Unaltered phenotypes may be observed, since off-target cleavage can occur in a non-coding region [55]. (2) The researchers tend to focus on the P_1 founders with intended insertions rather than those harboring possible off-target mutations [56–57]. (3) Most studies using animal models do not use genome-wide methodologies for detecting off-target cases, which could conceal some infrequent off-target editing sites [50]. In the same case, except for *lh* mutations, we did not conduct a thorough detection of all off-target individuals, due to it being time-consuming and expensive. Nevertheless, this does not preclude us from keeping the non-analyzed off-target individuals, as we will eventually genotype them in a genome-wide and unbiased way.

The pivotal role of LH in regulating reproduction in channel catfish is evident from our gene expression study. LH plays a crucial role in the final oocyte maturation and ovulation processes in females, as supported by significantly lower *lh* expression in *lh*-knockout catfish females when compared with controls. Conversely, we observed no substantial alterations in *lh* expression in *lh*-knockout males, underscoring the comparatively lesser role of LH in male reproduction. Our observations resonate with similar studies conducted in other species, such as zebrafish [22]. In *lhb*-mutant female zebrafish, sterility is a recurring outcome, further reinforcing the critical involvement of LH in female reproduction. However, *lhb*-mutant female zebrafish were fertile, as evidenced in the reduced Gonadosomatic Index (GSI) observed through morphological and histological analyses in *lhb* mutant males, and there were no discernible differences in sperm motility and testicular structure when comparing *lhb* mutants with control male zebrafish [22]. Therefore, our findings affirm the pivotal role of *lh* in triggering final oocyte maturation and ovulation in female channel catfish, while indicating its lesser significance in male reproduction. These results also raise the intriguing possibility of follicle-stimulating hormone signaling as a fish's predominant regulator of spermatogenesis, which needs further investigation.

Genetic mosaicism has been and remains another small obstacle. In this study, we failed to effectively obtain 100% of individuals without mosaics. In essence, mosaicism from CRISPR/Cas9-genome-edited organisms is common in the case of fertilized-egg-based editing, and mosaic animals have been observed in mice [58,59], rats [57], and zebrafish [60,61], with a variety of frequencies. CRISPR/Cas9 engineered mosaicisms bring undesired consequences, hindering the generation of homozygous positive offspring and prolonging the generation of homozygotes. We evaluated the expression of *As-Cath* transgene from five on-target positive P_1 founders and found that one individual had no expression in the gonad. In our study, several mosaic events were determined in the germline, resulting in the inability to transfer the *As-Cath* gene to the offspring. Thus, we believe that mosaicism is also common and unavoidable in non-model fish. Although early sperm/testis or egg/ovary genotyping can be effective in avoiding the cre-

ation of undesirable offspring, it is challenging to access the germline DNA without sacrificing the parents. However, homozygosity should be achieved in the F_2 and F_3 progeny. New strategies—that is, *Easi*-CRISPR, C-CRISPR [6], CRISPR/Cas9 HITI [46], and dCas9-SSAP [47]—might be used to reduce the induction of mosaic animals.

Regardless of the type of CRISPR/Cas9-mediated genome editing, microinjection always has irreversible effects on embryos; in our current study, these were increased mortality and decreased hatchability. High embryonic deaths were observed from sham- and CRISPR/Cas9-mediated-microinjection in our study, revealing that major mortality occurs due to the injection of the yolk, while fewer impacts stem from the DNA donors and reagents [44]. Although a high dosage resulted in a high embryonic mortality and lower hatching rate, it did not significantly reduce the fry survival rate compared with the WT group, which is in agreement with the findings by 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 the microinjection of about 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 significant growth depression or improvement in various families of P_1 founder. Nonetheless, the preliminary data revealed that the LH^-_As-$Cath^+$ fish had a greater gain in BW compared with the WT individuals after a 3-month culture in the tank, and the growth differences are emerging in the F_1 progeny. Our hypothesis is that the AMP transgenics should be healthier, allowing faster growth under certain culture conditions or life stages. cfGnRH-deficient channel catfish did not show significant effects in growth and survival throughout a 4-year culture compared with WT fish [19]. However, potential pleiotropic effects could exist when the *lh* gene is replaced by *As-Cath*, as in our case. Therefore, P_1 mosaic founders carrying the *As-Cath* transgene should be used to produce homozygous families; then, comparisons of the growth, survival rate, seinability, and carcass traits could be performed to make the enhanced performance of the LH^-_As-$Cath^+$ 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 have been achieved via NHEJ-mediated KO [18]. In addition, few studies have proved that gene-mutants can induce disease-resistant fish lines via KO to date [14]. In contrast, the integration of AMG is encouraging as a means of improving resistance against pathogens in fish [14,17]. Consumers generally have relatively little understanding of transgenesis and have more negative attitudes toward transgenic organisms than gene-edited organisms [62]; however, there is still some public pushback against transgenic/gene-edited animals that 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 the gut will digest most proteins and inactivate them even when consumed raw; ② since cooking denatures proteins and the proteins are further digested in the stomach, amino acids rather than active proteins are absorbed by humans; and ③ even though 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 to the 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 ecosystems [17]. Reproductive sterility via genome editing has been attracting research attention and offers opportunities to reduce environmental risks in aquaculture [62]. Moreover, representative examples have illustrated that reproductive confinement is promising in model and cultured fish by knocking out/disrupting reproduction-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 $IU{\cdot}kg^{-1}$ HCG coupled with 50 $\mu g{\cdot}kg^{-1}$ LHRHa restored fecundity at the highest level in comparison with other hormone treatments; however, this improvement was not significantly different from the improvement observed with a dose of 1200 $IU{\cdot}kg^{-1}$ HCG. 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.

Both on-target and off-target *As-Cath* transgenic catfish were established in the current study. It should be noted that biosafety is crucial when using gene editing/transgenesis in fish aquaculture. Key safety concerns include the comprehensive analysis of off-target effects through genome-wide assessments using advanced sequencing. In addition, assessing the stability of the genetic modification over generations is vital, achieved by tracking progeny to ensure that the intended changes persist and do not lead to unintended physiological or biochemical consequences due to alterations at non-target loci. Furthermore, since AMG transgenic fish produce AMPs as part of their genetic manipulation, the impact on the health of the host fish should be fully investigated. This includes assessing whether the peptides affect the fish's growth, immune system, and overall well-being. If these fish are intended for human consumption in the future, thorough safety assessments will be required. These will involve evaluating whether the AMPs or any other components of the transgenic fish pose a health risk to consumers. Special attention should be paid to allergenic potential and possible effects on the human gut microbiome.

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 sterile and environmentally sound fish lines with enhanced disease resistance. Encouragingly, the 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 the environmental dangers posed by escaped genetically modified individuals. We contend that the genome-editing tool should be used as a complement to existing breeding techniques, rather than as a replacement for them. Hence, a combination of genome editing and conventional selective breeding is required to maximize the benefits of CRISPR/Cas9 tools 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 engineering in catfish, which may provide a strategy for decreasing bacterial disease problems in aquaculture while simultaneously reducing environmental risks. Reproduction was successful but reduced compared with the WT; thus, hormone therapy to restore fertility requires further research and improvement.

CRediT authorship contribution statement

Jinhai Wang: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Validation, Writing – original draft. **Baofeng Su:** Conceptualization, Funding acquisition, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Validation, Project administration, Supervision, Writing – review & editing. **De Xing:** Conceptualization, Data curation, Investigation, Methodology. **Timothy J. Bruce:** Methodology, Writing – review & editing. **Shangjia Li:** Data curation, Investigation. **Logan Bern:** Data curation, Investigation. **Mei Shang:** Data curation, Investigation, Writing – review & editing. **Andrew Johnson:** Data curation. **Rhoda Mae C. Simora:** Data curation. **Michael Coogan:** Data curation. **Darshika U. Hettiarachchi:** Data curation. **Wenwen Wang:** Data curation. **Tasnuba Hasin:** Data curation. **Jacob Al-Armanazi:** Data curation. **Cuiyu Lu:** Data curation. **Rex A. Dunham:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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Compliance with ethics guidelines

Jinhai Wang, Baofeng Su, De Xing, Timothy J. Bruce, Shangjia Li, Logan Bern, Mei Shang, Andrew Johnson, Rhoda Mae C. Simora, Michael Coogan, Darshika U. Hettiarachchi, Wenwen Wang, Tasnuba Hasin, Jacob Al-Armanazi, Cuiyu Lu, and Rex A. Dunham declare that they have no conflict of interest or financial conflicts to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eng.2023.12.005.

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