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1 **Generation of eco-friendly channel catfish, *Ictalurus punctatus*, harboring**
2 **alligator cathelicidin gene with robust disease resistance by harnessing different**
3 **CRISPR/Cas9-mediated systems**
4
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15

16 **Abstract**

17 The CRISPR/Cas9 platform holds promise for modifying fish traits of interest as a
18 precise and versatile tool for genome manipulation. To reduce introgression of
19 transgene and control reproduction, catfish species have been studied for upscaled
20 disease resistance and intervening of reproduction to lower the potential
21 environmental risks of introgression of escapees' as transgenic animals. Taking
22 advantage of the CRISPR/Cas9-mediated system, we succeeded in integrating the
23 cathelicidin gene from an alligator (*Alligator sinensis*; *As-Cath*) into the target
24 luteinizing hormone (*LH*) locus of channel catfish (*Ictalurus punctatus*) using two
25 delivery systems assisted by double-stranded DNA (dsDNA) and single-stranded
26 oligodeoxynucleotides (ssODNs), respectively. In this study, high knock-in (KI)
27 efficiency (22.38%, 64/286) but low on-target was achieved using the ssODN strategy,
28 whereas adopting a dsDNA as the donor template led to an efficient on-target KI
29 (10.80%, 23/213). On-target KI of *As-Cath* was instrumental in establishing the *LH*
30 knockout (LH^{-}_As-$Cath^{+}$) catfish line, which displayed heightened disease resistance
31 and reduced fecundity compared to the wild-type sibling fish. Furthermore,
32 implanting with HCG and LHRHa can restore the fecundity, spawnability and
33 hatchability of the new transgenic fish line. Overall, we replaced the *LH* gene with an
34 alligator cathelicidin transgene and then administered hormone therapy to gain
35 complete reproductive control of disease-resistant transgenic catfish in an
36 environmentally sound manner. This strategy not only effectively improves the
37 consumer-valued traits, but also guards against genetic contamination. This is a

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38 breakthrough in aquaculture genetics to confine fish reproduction and prevent the
39 establishment of transgenic or domestic genotypes in the natural environment.
40

41 **Keywords**
42 Genome editing, ssODN, dsDNA, antimicrobial peptide, immune, reproductive
43 confinement, aquaculture
44
45

46 1. Introduction

47 Innovative biotechnologies continuously develop as science advances, benefiting food
48 production, quality as well as animal and human welfare. Since its inception,
49 CRISPR/Cas9 (clustered regularly interspaced short palindromic
50 repeats/CRISPR-associated protein 9) has served as a prototype in genome
51 engineering, paving the way for new possibilities in transgenesis and breeding. Two
52 mechanisms are involved for DNA repair when double strand breaks are induced by
53 the CRISPR/Cas9 complex: non-homologous end joining (NHEJ) and
54 homology-directed repair (HDR) [1]. Both mechanism-mediated strategies have been
55 employed in aquaculture to improve the consumer-valued qualities targeted within
56 genetic breeding programs. These harness the NHEJ repair pathway to knock out
57 (KO)/disrupt functional genes or knock in (KI) exogenous genes of interest via HDR
58 at the expected locus to improve the target traits.
59

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

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72 model, zebrafish (*Danio rerio*), and sequencing results revealed that erroneous repair
73 was more likely to occur when ssODNs were used as repair templates. Alternatively,
74 the modified donor plasmid containing two HAs flanked by two single guide
75 (sgRNA)-targeted sequences (double-cut donors) typically results in a site-specific KI
76 with a high integration rate [8,9], and this HA-medicated KI has been adapted to
77 zebrafish and medaka (*Oryzias latipes*) [9,10]. Theoretically, if we directly offer a
78 linear double-stranded DNA (dsDNA) flanked by two HAs derived from 5'- and 3'-
79 ends of the targeted site and ignore the difference in stability between circular DNA
80 and dsDNA donors, the KI efficiency will increase by convention. In addition to the
81 type of donors, a proper concentration of each component of the CRISPR/Cas9
82 system has a great positive impact on KI by reducing off-target events and embryo
83 lethality. In this regard, we anticipate achieving extremely efficient KI if a reliable
84 delivery system and an optimized dosage of components are chosen in a non-model
85 fish species.
86
87 Currently, transgenesis and CRISPR/Cas9-mediated genome editing have
88 revolutionized traditional theories to accelerate the pace of aquaculture breeding
89 programs, and delivered edible commercial products, such as the genetically modified
90 AquAdvantage salmon [11,12], gene-edited tiger puffer fish and red sea bream
91 (https://doi.org/10.1038/s41587-021-01197-8, 2022). Although the NHEJ strategy
92 predominates in altering the consumer-focused traits of fish species, including growth,
93 coloration, and reproduction, the HDR-mediated KI is an effective way to improve the
94 omega-3 fatty acid content and disease resistance [13-15]. In comparison to the
95 non-insertion of KO mutations, the integration of foreign genes by harnessing the
96 HDR pathway usually raises concerns about low KI efficiency and introgression,
97 which directly impact the advocacy of this method and the consumer acceptance of
98 gene-inserted fish [16]. As a result, it is imperative to devise a strategy for both
99 improving the desired traits and preventing introgression to alleviate public concerns
100 about gene-inserted animals. Fortunately, numerous genome-editing-based studies
101 have demonstrated that it is possible to render fish reproductively sterile by
102 altering/disrupting key genes involved in reproduction via the NHEJ repair pathway.
103 Thus, potentially reducing negative environmental effects associated with genetically
104 modified fish [17-19]. Luteinizing hormone (*LH*) gene regulates gametogenesis and
105 gestation through binding the receptor [20,21]. LH-deficient female zebrafish are

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106 infertile, whereas the mutant males are fertile, indicating that the *LH* gene facilitates
107 fish oocyte maturation and triggers ovulation [22]. In addition, interruption of the *LH*
108 gene in channel catfish and white-edged rockfish (*Sebastes taczanowskii*) can result in
109 the production of sterile lines [21,23].
110
111 Large-scale disease outbreaks are inevitable, and methods of disease control need to
112 be improved. Antimicrobial peptides (AMPs) are polypeptides that serve as
113 substitutes for antibiotics in a variety of species' initial line of defense (innate
114 immunity) against microbial invasions without developing considerable antibiotic
115 resistance [24,25]. AMPs and antimicrobial peptide genes (AMGs) including cecropin,
116 hepcidin, piscidin, epinecidin-1, lysozyme, and lactoferrin have been used for decades
117 to improve disease resistance in a variety of aquatic animals, as feed supplements or
118 transgenes [13,26]. Cathelicidins are a particularly important AMP family, sharing the
119 common cathelin-like domain [27] and exhibiting broad-spectrum antimicrobial and
120 immune-modulating activities [28]. Recent investigations have shown that
121 alligator-derived cathelicidin inhibits fish pathogens both *in vivo* and *in vitro* [29-31].
122 Therefore, integrating the AMG into the genomic DNA has broad prospects for
123 establishing novel disease-resistant fish lines.
124
125 Fish transgenic for AMGs could provide a significant option to address disease
126 problems, however, and additional goal would be to prevent the possibility of
127 breeding of escapees with wild populations. Hypothetically, a reproductive gene such
128 as *LH*, responsible for gametogenesis and gestation could be knocked out at the DNA
129 level with the replacement of a cathelicidin gene, leading to sterile fish with
130 heightened disease resistance. Genome-edited sterilized fish from this approach would
131 have fertility temporarily restored with hormone therapy used for artificial spawning
132 of fish, and it is achievable to produce environmentally-compatible and
133 disease-resistant fish lines. In this study, two CRISPR/Cas9 delivery systems: HA-
134 and ssODN-mediated KI were employed to insert the *As-Cath* gene at the channel
135 catfish (*Ictalurus punctatus*) *LH* locus to develop a reversibly sterile and
136 disease-resistant line. We compared the KI efficiency, hatchability and fry survival
137 from various systems, and then restored the fertility of As-Cath-integrated sterile of P_0
138 founders through hormone therapy. In addition, the bacterial resistance of P_0 and F_1
139 individuals from the new fish line was further evaluated.

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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

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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, Benching, 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 ℃ 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 ℃ 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.

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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

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242 from the same parents, and the microinjection was terminated after 90 min
243 post-fertilization.
244
245 All injected and control embryos were transferred into 10-L tubs filled with 7-L
246 Holtfreter's solution (59 mmol NaCl, 2.4 mmol $NaHCO_3$, 1.67 mmol $MgSO_4$, 0.76
247 mmol $CaCl_2$, 0.67 mmol KCl) [37] and 10－12 ppm doxycycline for hatching
248 immediately after microinjection. All tubs were placed in the same flow-through
249 hatching trough and a heater was put upstream of the trough to ensure that the water
250 temperature was 26－28 ℃ while dissolved oxygen levels were > 5 ppm via
251 continuous aeration with airstones. Holtfreter's solution was replaced twice per day
252 and dead embryos/fry were collected and recorded daily during hatching to analyze
253 hatchability, fry survival rate and genotype. The hatched fry were transferred to a
254 Holtfreter's solution without doxycycline and fed with live *Artemia* nauplii four times
255 per day. After one week of culture in tubs, all fry from each treatment were stocked
256 separately into 60 L aquaria (120 fish/tank) in a recirculating system for growth
257 experiments. Feed pellet size was adjusted according to the size of the fish's mouth as
258 the fish grew. In detail, fry in tanks fed with Purina® AquaMax® powdered feed (50%
259 crude protein, 17% crude fat, 3% crude fiber, and 12% ash) four times per day for two
260 months. Then fingerlings were fed with Aquaxcel WW Fish Starter 4512 (45% crude
261 protein, 12% crude fat, 3% crude fiber, and 1% phosphorus) twice a day for two
262 months. Juvenile fish were fed with WW 4010 Transition feed (40% crude protein, 10%
263 crude fat, 4% crude fiber, and 1% phosphorus) once a day [14]. All fish were fed to
264 satiation.
265
266 *2.5. Integration analysis and mutation detection*
267
268 After a 4-month culture, all fingerlings (20－40 g) were pit-tagged (Biomark Inc.,
269 Boise, Idaho, USA) to distinguish each individual, the fish from different treatments
270 were then mixed together and randomly dispersed into two circular tanks (1,200 L
271 volume filled with ~800 L of water) with the same density (120 fish/tank) for growth
272 comparison monthly. Meanwhile, the pelvic fin clip and barbel were taken from
273 anesthetized fish for DNA extraction and genotypic identification. During this phase,
274 all fish received WW 4010 Transition feed once a day to satiation. Different
275 genotyping strategies were involved for these two constructs:

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276 ssODN1_As-Cath_ssODN2, the CDS region of As-Cath was amplified to confirm
277 gene insertion using primers Cath1-F/R (forward and reverse), and the promoter
278 region was amplified via primers Prom1-F/R. As for the junctions, ssODN1 and
279 ssODN2 regions were amplified using primers ssODN1-F/R and ssODN2-F/R to
280 determine whether it was a target-site insertion. With respect to the
281 HA1_As-Cath_HA2 construct, the As-Cath and promoter regions were detected using
282 primers Cath2-F/R and Prom2-F/R, respectively. Then the left HA and right HA
283 junctions were amplified via primers HA1-F/R and HA2-F/R. Primers were designed
284 using the online software Primer3Plus
285 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and listed in
286 Table S1 (Appendix B). PCR was performed in a 10-μL system and PCR products
287 were resolved and visualized by running 1.0% agarose gel with 1 × tris-acetate-EDTA
288 (TAE) buffer, and a bright band of each region with the corresponding length
289 indicated an on-target positive (LH^{-}_As-$Cath^{+}$). Here, if we can determine that some
290 individuals have been inserted with the As-Cath fragment, but we can not detect the
291 junctional regions (HA- or ssODN-region), we then conclude them as potential
292 off-target positives (LH^{+}_As-$Cath^{+}$).
293
294 With respect to the LH^{+}_As-$Cath^{+}$ fish, we selected 60 individuals to be tested for *LH*
295 mutations. In this case, PCR was performed in a 20 μL-volume system using Expand
296 High Fidelity^{PLUS} PCR System (Roche Diagnostics, Indianapolis, IN, USA) according
297 to Elaswad et al. [35], and LH-F/R primers were used in both constructs. Then, the
298 surveyor mutation detection assay was performed via Surveyor Mutation Detection
299 Kit (Integrated DNA Technologies, IDT, Coralville, Iowa, USA) according to the
300 detailed instructions [38]. A negative control reaction was included in the assay by
301 using genomic DNA from the nCT group. Surveyor-digested DNA samples were
302 electrophoresed for 1 hour in a 2% agarose gel using 1 × TBE buffer and compared to
303 wild-type samples.
304
305 *2.6. DNA sequencing*
306
307 For the integrated As-Cath, promoter and junction sequences, PCR of positive
308 samples was performed in a 50 μL-volume of system. Then the PCR products were
309 purified using the QIAquick^{R} PCR Product Purification Kit (QIAGEN, Hilden,

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310 Germany) according to the manufacturer's instructions. Before sequencing, all
311 purified DNA samples were quantitated and identified using Nanodrop and by
312 running 1.0% agarose gel. Primers Cath1-F/Cath2-F and Prom1-F/Prom-2F were used
313 for sequencing of As-Cath and promoter regions for HA1_As-Cath_HA2 and
314 ssODN1_As-Cath_ssODN2 constructs, respectively; primers HA1-F/HA2-F and
315 ssODN1-F/ssODN2-F were used for sequencing of junctional regions for these two
316 constructs, respectively.
317
318 Regarding LH mutations, we cloned the PCR products of putative mutant individuals
319 using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) before sequencing following
320 the instructions with some modifications. Briefly, PCR was performed on each mutant
321 individual that was previously identified with Surveyor assay using the primers
322 LH-F/R for the next cloning steps. In addition, the DNA of three wild-type individuals
323 from the nCT group was prepared using the same primers and procedures, then
324 combined into one reaction and cloned as a wild-type control for sequencing. After
325 cloning, we transformed the pCR™4-TOPO vector containing the PCR products into
326 One Shot TOP10 Electrocomp™ *E. coli* (Invitrogen, Carlsbad, CA) as previously
327 described [35]. Then 15 single colonies were randomly picked up to perform Colony
328 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
332 Five 12-month-old on-target positive fish and five sham-injected control fish were
333 randomly chosen and sacrificed. Fourteen tissues, including skin, liver, kidney, spleen,
334 blood, intestine, gill, stomach, fin, barbel, muscle, eye, brain and gonad of each
335 individual were collected in 1.5 mL tubes and immediately transferred into liquid
336 nitrogen for DNA and RNA isolation. PCR and quantitative real-time PCR (qRT-PCR)
337 were conducted to detect the *As-Cath* gene's potential mosaicism and mRNA level.
338 Total RNAs were isolated from various tissues using TRIzol reagent (Thermo Fisher
339 Scientific) and were reverse transcribed to cDNA using iScript™ Synthesis Kit
340 (Bio-Rad, Hercules, CA) following the manufacture protocols.
341
342 qRT-PCR was performed on a C1000 Thermal Cycler using SsoFast™ EvaGreen
343 Supermix kit (Bio-Rad, Hercules, CA) according to the instructions. Concentrations

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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_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_0 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_0 individuals are expected to reach sexual maturity [40]. To evaluate the reproduction of two-year-old KI founders, on-target positive (LH^-_As-$Cath^+$), off-target positive (LH^+_As-$Cath^+$), and wild-type (WT) fish were selected to conduct a three-round mating experiment. Firstly, 3 pairs of WT, 6 pairs of LH^-_As-$Cath^+$, and 4 pairs of LH^+_As-$Cath^+$ mature parents were randomly placed into 13 tanks ($60 \times 45 \times 30$ cm^3) 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^-_As-$Cath^+$ females were fertile. After this period, we recruited 6 more pairs of LH^-_As-$Cath^+$ 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_1 fish

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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_1
381 individuals of each genotype except the control groups. The same culture and
382 genotyping procedures as described above were applied to the F_1 generation.
383
384 *2.10. Experimental challenge with Flavobacterium covae 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_0 and F_1 fish were challenged by *F.*
389 *covae* and *E. ictaluri.*
390
391 *F. covae* challenge. Healthy P_0 fingerlings with body weight 150.62 ± 4.24 g (mean ±
392 SEM), including four genotypes (15 fish/genotype): LH^-_As-$Cath^+$, LH^+_As-$Cath^+$,
393 negative LH^+_As-$Cath^-$ (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. covae* 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_{540} = 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 (4.55×10^8 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: 2.46×10^6
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

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412 of rearing water for 1.5 hours with sterile modified Shieh broth (325 mL) instead of
413 the bacterial culture. With respect to the challenge of F_1 fry (3.15 ± 0.24 g), four
414 families of F_1 fry (45 fish/family): LH^-_As-$Cath^+$, LH^+_As-$Cath^+$, LH^+_As-$Cath^-$ and
415 WT were selected, and each family was randomly divided into three replicates with
416 15 fish per basket. The same challenge procedure and strain of *F. covae* with a dose of
417 4.75×10^8 CFU/mL (immersion dose: 2.57×10^6 CFU/mL) were implanted for the F_1
418 generation.
419
420 *E. ictaluri* challenge. Sixty P_0 fish (142.62 ± 3.72 g) including the above four
421 genotypes, were prepared for the *E. ictaluri* challenge. *E. ictaluri* (S97-773) was
422 provided by the USDA-ARS, Aquatic Animal Health Research Unit, Auburn, AL. The
423 detailed procedures of the *E. ictaluri* challenge were performed according to Simora
424 et al. [30] with some modifications. Briefly, 1 mL of frozen glycerol stock of *E.*
425 *ictaluri* was inoculated into 20-mL brain–heart infusion broth (BHIB; Hardy
426 Diagnostics) at 26°C in a shaker incubator at 180 rpm for 24 hours. And then bacteria
427 were subcultured into 1-L BHIB for another 24 hours at the same condition until the
428 cell density reached ~1×10^8 CFU/mL based on the OD_{600} value. All 60 P_0 individuals
429 were transferred into one 1,800-L tank for the challenge. Before starting *E. ictaluri*
430 infection, water was lowered to a total of 100 L, then one liter of *E. ictaluri*
431 suspension containing 3.2×10^8 CFU/mL cells was added to the tank resulting in a
432 final immersion dose of 3.2×10^6 CFU/mL. Fish were immersed statically for 2 hours
433 with aeration > 5 ppm, then water was restored. In addition to infected groups, one
434 control tank containing 30 fish received only BHIB as a mock-challenged group. With
435 respect to the challenge of F_1 fingerlings (54.27 ± 1.49 g), a total of four genotypes
436 containing 60 fish were selected, and the same challenge procedure and strain of *E.*
437 *ictaluri* with a dose of 2.8×10^8 CFU/mL (immersion dose: 2.8×10^6 CFU/mL) were
438 implanted for the F_1 generation.
439
440 During the first 72 h of the experiment, we checked for mortality every four hours and
441 then three times daily. Challenged fish were continuously monitored for 10 days for
442 external clinical signs of *F. covae*/*E. ictaluri* and confirmation of bacteria colony
443 growth by isolating bacteria from the kidney and liver to determine the cause of death,
444 and dead individuals were recorded over time.
445

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446 *2.11. Statistical analysis*

447

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

459

460 **3. Results**

461

462 *3.1. Targeted KI of As-Cath gene into the LH locus*

463

464 Both the 2H2OP and dsDNA systems can induce As-Cath-integrated catfish lines with
465 high integrated ratios, but the 2H2OP system had significant off-target effects (Fig.
466 1(CD), Fig. S1-S4 in Appendix B). More specifically, the 2H2OP system containing
467 50 ng/µL of donors (2H2OP50) showed the highest KI efficiency at 27.61% (37/134),
468 followed by the groups 2H2OP100 (17.76%, 27/152), dsDNA50 (12.21%, 26/213)
469 and dsDNA100 (10.25%, 25/244) (Table S2 in Appendix B). Although the 2H2OP50
470 group can introduce the highest KI efficiency ($P < 0.01$) (Fig. 2(A)), and 2H2OP
471 system or 50 ng/µL of donors bring a significantly higher KI efficiency than the
472 dsDNA method ($P = 0.0001$) or 100 ng/µL of donors ($P = 0.00469$) (Fig. 2(BC)).
473 However, the dsDNA with 50 ng/µL donors demonstrated the highest on-target KI
474 efficiency (10.80%, 23/213) compared to other treatments ($P < 0.01$) (Fig. 2(D)). In
475 contrast, only one on-target KI case was observed in the 2H2OP system, which was
476 significantly lower than that in the dsDNA ($P < 0.0001$) (Fig. 2(E)). Although
477 different dosages of donors exhibited a significant effect on the total KI efficiency, our
478 results indicated that this difference was not significant in the on-target KI ($P =$
479 0.3577) (Fig. 2(F)).

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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 surpriority 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*

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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^-_As-$Cath^+$ 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^-_As-$Cath^+$ 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^+_As-$Cath^+$ 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^-_As-$Cath^+$ pairs (0%). Compared to the LH^-_As-$Cath^+$
541 pairs, WT and LH^+_As-$Cath^+$ fish had higher spawnability under natural pairing
542 conditions (P = 0.0148 and P = 0.1743). In addition, the LH^+_As-$Cath^+$ 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^-_As-$Cath^+$ females to give eggs, indicating

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548 LH-deficient females blocked oocyte maturation and ovulation. However, our results
549 discovered that a combination of LHRHa and HCG can effectively induce spawning
550 for the LH^{-}_As-$Cath^{+}$ females when both males and females were primed.
551 Specifically, two, two and one female gave eggs after 24 to 48 hours post-hormone
552 injection from the 1200 IU (6213 eggs/BW), 1600 IU (5514 eggs/BW) and 2000
553 IU/kg (3778 eggs/BW) HCG group combined with 50 μg/kg LHRHa, respectively.
554 These three treatments significantly improved the fecundity compared to 0.85 % NaCl
555 injection ($P < 0.0001$). Additionally, the fecundity decreased with increasing hormone
556 dosage, but the difference among these three hormone dosages was not significant (P
557 $= 0.0731$). Nevertheless, the fecundity can be restored to a normal level when 1200 (P
558 $= 0.2627$) or 1600 ($P = 0.1983$) IU/kg HCG combined with 50 μg/kg LHRHa was
559 adopted (Fig. 4(C)). Compared with the WT and the other hormonal-therapy groups,
560 the 2000 IU/kg HCG group significantly reduced the fecundity (3778 eggs/BW, $P =$
561 0.0494) and hatchability (18.01%, $P = 0.0476$) (Fig. 4(D)). Although different
562 hormonal treatments had varying effects on fecundity and hatchability, they had no
563 effects on fry survival at the early stage ($P = 0.1018$) (Fig. 4(E)).
564

565 *3.5. F_1 genotyping, growth comparison in P_0 and F_1*

566

567 As mentioned above, three WT, two LH^{+}_As-$Cath^{+}$, and five LH^{-}_As-$Cath^{+}$ families
568 were generated from our three-round mating experiment. However, genotype analysis
569 determined that only one family in the LH^{+}_As-$Cath^{+}$ line (33.33%[10/30] integrated
570 rate in the F_1 offspring) and two families in the LH^{-}_As-$Cath^{+}$ line (40%[12/30]
571 integrated rate in the F_1 progeny of family 1 and 46.67%[14/30] integrated rate in the
572 F_1 offspring of family 2), respectively, had the *As-Cath* gene detectable in the F_1
573 generation. These results further confirmed the existence of the mosaic phenomenon
574 in the P_0 founders.
575

576 To determine the effects of *LH* disruption and *As-Cath* integration on fish growth, we
577 compared the BW over time of the P_0 founders and the F_1 progeny, respectively. The
578 growth data suggested that the LH^{-}_As-$Cath^{+}$ individuals did not show superiority in
579 terms of growth in the first nine months in the P_0 generation. Nonetheless, P_0
580 LH^{-}_As-$Cath^{+}$ fish exhibited the largest body gain (36.35 g) compared to other
581 genotypes (25 g). Furthermore, significantly faster growth was demonstrated in the F_1
582 generation of LH^{-}_As-$Cath^{+}$ after a three-month culture. Hence, our results indicated

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583 more immediate growth potential for the LH$^-$_As-Cath$^+$ 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_0 and F_1 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$^+$_As-Cath$^-$) in both P_0 (13.33% *vs* 20%, $P = 0.8682$) and F_1 generation (26.67% *vs*
593 40%, $P = 0.8955$). However, LH$^-$_As-Cath$^+$ and LH$^+$_As-Cath$^+$ fish exhibited
594 significantly improved survival post *F. covae* infection compared to the WT control
595 group in both P_0 founders (LH$^-$_As-Cath$^+$ *vs* WT: 73.33% *vs* 13.33%, $P = 0.0016$;
596 LH$^+$_As-Cath$^+$ *vs* WT: 66.67% *vs* 13.33%, $P = 0.0014$) and F_1 progeny
597 (LH$^-$_As-Cath$^+$ *vs* WT: 86.67% *vs* 26.67%, $P = 0.0010$; LH$^+$_As-Cath$^+$ *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_0, and
601 86.67% *vs* 73.33%, $P = 0.3613$ for F_1). Furthermore, our findings revealed that the F_1
602 progeny was more resistant to *F. covae* than its P_0 parents (Fig. 5(AB)).
603
604 Increased resistance to *E. ictaluri* was also observed in the P_0 (LH$^-$_As-Cath$^+$ *vs* WT:
605 73.33% *vs* 33.33%, $P = 0.0125$; LH$^+$_As-Cath$^+$ *vs* WT: 60% *vs* 33.33%, $P = 0.0427$)
606 and F_1 generations (LH$^-$_As-Cath$^+$ *vs* WT: 66.67% *vs* 40%, $P = 0.0558$;
607 LH$^+$_As-Cath$^+$ *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_0; 70% *vs* 40%, $P = 0.0335$ for F_1). Nevertheless, there was
611 no significant difference in LH$^-$_As-Cath$^+$ and LH$^+$_As-Cath$^+$ fish (73.33% *vs* 60%, P
612 $= 0.4566$ for P_0; 66.67% *vs* 73.33%, $P = 0.6851$ for F_1) (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

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618 impact of transgenic fish on the ecosystems and genetic biodiversity. Specifically, we
619 successfully integrated an AMG into the reproduction-associated locus using different
620 CRISPR/Cas9-mediated systems. We identified a suitable KI system for channel
621 catfish to achieve boosted resistance against fish pathogens and reproductive control,
622 reducing the reliance on antibiotics and anti-parasitics in aquaculture. The
623 HA-mediated CRISPR/Cas9 system displayed a high integrated rate, low off-target
624 events, and low toxicity. In addition, reproduction is entirely controllable and can only
625 be restored to normal levels of fecundity with hormone therapy in the new fish line. In
626 general, the insertion of the cathelicidin gene at the *LH* locus for enhanced resistance
627 against infectious diseases and reproductive confinement to improve consumer-valued
628 qualities and to promote the environmental friendliness of transgenic fish appears
629 promising.
630

631 There have been several obstacles involved in the CRISPR/Cas9-mediated KI system
632 when it is used in the embryos of non-model animals. In the history of genome editing,
633 the initial CRISPR/Cas9 systems were proposed based on mammalian cells or
634 embryos of the model animals. From model to non-model animals, there are several
635 uncertainties, such as embryo size, developmental period, and the sensitivity to Cas9
636 protein that researchers have to optimize a fitted system when starting a new species'
637 genome editing. Yoshimi et al. [5] demonstrated that the ssODN-mediated end joining
638 approach induced a high integrated rate of 17.6% (3/17) in rats when a short ssODN
639 template was provided. Conversely, recent works indicated that ssODN-mediated KI
640 could induce a high percentage (17.8%) of indel mutations in sheep [41]. In the
641 current study, we used CRISPR/Cas9 systems mediated by ssODN and HA to create
642 on-target KIs of the *As-Cath* gene at the *LH* locus. Although a high KI efficiency of
643 22.38% (64/286) was detected in the ssODN-mediated system, it caused a high
644 off-target frequency (> 90%) in the channel catfish. Our results are in agreement with
645 findings in zebrafish, which have illustrated that erroneous ssODN integration
646 occurred when various template lengths were adopted [7]. These studies suggest that
647 ssODN-mediated KI efficiency in fish models relies heavily on ssODN templates [42],
648 and caution is warranted when employing ssODNs to create KI models.
649

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

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653 with a linear dsDNA donor displayed a total integrated rate of 29% at the non-coding
654 region of channel catfish genome, which is drastically higher than that of this work
655 (29% *vs* 11.16%[51/457]). We believe this difference in integration rate is due to the
656 different sample sizes, unknown functions in the target regions (non-coding *vs* *LH*
657 locus), efficiency of sgRNA and HA, and unpredictable genetic interaction; the larger
658 sample size from our study could give more robust conclusions. These findings reveal
659 that the HA-mediated system is more effective in the catfish species compared to the
660 ssODN. The KI efficiency of HDR-induced CRISPR/Cas9 has been at a low level
661 including in cell lines and model animals [5,7,9]. Fortunately, new
662 CRISPR/Cas-mediated techniques are constantly being developed. For instance, the
663 CRISPR/Cas12i-mediated system shows promise in multiplexed genome editing with
664 high mutation rates in human T cells [45]. Additionally, Kelly et al. [46] established a
665 CRISPR/Cas9 HITI system for the insertion of large DNA donors with high integrated
666 efficiency of 36% in human 293T cells. Recently, a new approach named
667 dCas9-SSAP demonstrated a high on-target KI efficiency (~20%) knocking in long
668 sequences in mammalian cells [47]. These new tools or systems are encouraging to be
669 applied from model to non-model animals and could improve genome-editing
670 efficiency.
671

672 Although we predicted and avoided possible off-target sites using the
673 well-acknowledged software, the actual integration results showed the existence of
674 off-target activities. This is mainly due to the failure of *in silico* prediction to predict
675 *bona-fide* off-target sites *in vivo* [48,49]. Furthermore, the frequency of off-target
676 events is higher *in vivo* of animal experiments than that of in cellular experiments *in*
677 *vitro* [50]. The majority of published studies contend that the observed unintended
678 mutations/insertions is one major concern in the application of the CRISPR/Cas9
679 system, which could confound the interpretation of findings [49,51,52]. However,
680 although some reports claim that no detectable undesirable mutations/insertions from
681 the genotypes or phenotypes have been revealed in mice and fish [44,53,54], the
682 following underlying potentials could be noted: 1) Unaltered phenotypes may be
683 observed since the off-target cleavage can occur in a non-coding region [55]. 2) The
684 researchers tend to focus on the P_0 founders with intended insertions rather than those
685 harboring possible off-target mutations [56-57]. 3) Most published research using
686 animal models does not use genome-wide methodologies for detecting off-target cases,
687 which could conceal some infrequent off-target editing sites [50]. In the same case,

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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_0 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_2 and
709 F_3 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

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723 Elaswad et al. [35]. This may be because microinjection only has a detrimental effect
724 on the yolk of the embryo. Still, this effect no longer affects the fry once the fertilized
725 eggs have successfully hatched. Given the unavoidable physical lethality of embryos,
726 off-target effects and mosaicisms, we recommend microinjection of ~3000 fertilized
727 eggs for non-model fish species in order to afford enough gene-edited fish for
728 subsequent validation experiments.
729
730 To assess the pleiotropic effects, we compared the growth performance of the
731 on-target/off-target As-Cath-integrated fish line with the WT population. Our findings
732 demonstrated that off-target insertions did not exhibit growth depression or
733 improvement in various families of P_0 founders. Nonetheless, the preliminary data
734 revealed the LH^-_As-$Cath^+$ fish had a greater gain in body weight compared to the
735 WT individuals after a three-month culture in the tank, indicating that the growth
736 differences are emerging in the F_1 progeny. This variation may be due to heterozygous
737 individuals lacking stable genetic traits, or off-target integrations in other regions
738 concealing growth advantages in the P_0 generation [50]. cfGnRH-deficient channel
739 catfish did not show significant effects in growth and survival throughout a four-year
740 culture compared to the WT fish [19]. However, potential pleiotropic effects could exist
741 when the *LH* gene is replaced by the As-Cath in our cases. Therefore, P_0 mosaic
742 founders carrying the *As-Cath* gene should be used to produce F_1, F_2 and F_3
743 homozygous families, and then the comparisons of the growth, survival rate, seinability
744 and carcass traits could be performed to avail the enhanced performance of
745 LH^-_As-$Cath^+$ fish line more transparent to farmers and the public in the future.
746
747 HDR-mediated KI is rarely applied in aquaculture due to the very low integration
748 efficiency, but most of the traits were achieved by NHEJ-mediated KO [17,18]. In
749 addition, few studies proved that gene-mutants can induce disease-resistant fish lines
750 via KO to date [13]. By contrast, the integration of AMG is encouraging to improve
751 resistance against pathogens in fish [13,16]. However, consumers generally have
752 relatively little awareness of transgenesis and have more negative attitudes toward
753 genetically modified organisms than genome-edited organisms [62], hence the public
754 pushback against transgenic/gene-edited animals is hindering them from reaching the
755 market. Here, we reasonably contend that cathelicidin transgenic catfish would not
756 pose a threat to food safety since: 1) Meat from artificially grown alligators is edible

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

763 Nonetheless, scientists and breeders need to be aware of the possible damage that
764 genetically modified fish could cause to the environment and ecosystem [16]. On the
765 one hand, reproductive sterility via genome editing has been attracting the attention of
766 researchers and offering opportunities to reduce environmental risks in aquaculture
767 [62]. On the other hand, representative examples have illustrated that reproductive
768 confinement is promising in model and cultured fish by knocking out/disrupting
769 gonadal development-related genes [23,63-66]. Recently, Qin et al. [19] demonstrated
770 that the reproduction-blocked channel catfish are sterile, and this reproductive
771 confinement can be lifted through hormone therapy with LHRHa. In this study, the
772 dose of 1600 IU/kg HCG coupled with 50 µg/kg LHRHa can restore fecundity at the
773 highest level in comparison to other hormone treatments, but this improvement is not
774 significant from that of 1200 IU/kg HCG. Therefore, a low dose of 1200 IU/kg HCG
775 is recommended for hormone therapy to restore the reproduction of the sterile fish
776 line to reduce costs. In addition to genetically achieving reproductive sterility,
777 well-confined culture systems should be adopted to avoid the escape of
778 mutant/transgenic individuals, especially in the experimental phase of transgenic fish.
779
780 **5. Conclusions**
781
782 We established a sterile catfish line that confers enhanced resistance to fish pathogens
783 by expressing the cathelicidin protein. Our study has demonstrated that the insertion
784 of the cathelicidin gene at the *LH* locus by harnessing the HA- or ssODN-mediated
785 CRISPR/Cas9 system can be a robust approach to produce sterilized and
786 environmentally-sound fish lines with enhanced disease resistance. Encouragingly,
787 CRISPR/Cas9-mediated KI of AMGs at the reproduction-related loci coupled with
788 hormone therapy could be applied in other commercial fish to increase profits and
789 lower environmental dangers posed by escaped genetic-modified individuals. Notably,
790 even though the desired traits (on-target insertions) can be quickly achieved through

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791 CRISPR/Cas9-mediated genome editing, this does not safeguard that we will be able
792 to yield enough non-mosaic P_0 founders. We contend the genome-editing tool should
793 be used as a complement to existing breeding techniques, not a replacement for them.
794 Hence, a combination of genome editing and conventional selective breeding is
795 required to maximize the benefits of CRISPR/Cas9 tools more effectively in aquatic
796 applications and to hasten the breeding process. In conclusion, this study showed the
797 potential of overexpressing a disease-resistant peptide inserted at a
798 reproduction-related gene using CRISPR/Cas9 in channel catfish, which may provide
799 a strategy of decreasing bacterial disease problems in catfish at the same time
800 reducing environmental risks.
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813 **Acknowledgments**
814
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817 and Alabama Agricultural Experiment Station grant (AAES-AIR). Jinhai Wang was
818 supported by a scholarship from the China Scholarship Council.
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823 **Appendix A. Supplementary data**
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825 Supplementary data to this article can be found from Appendix A and Appendix B.
826

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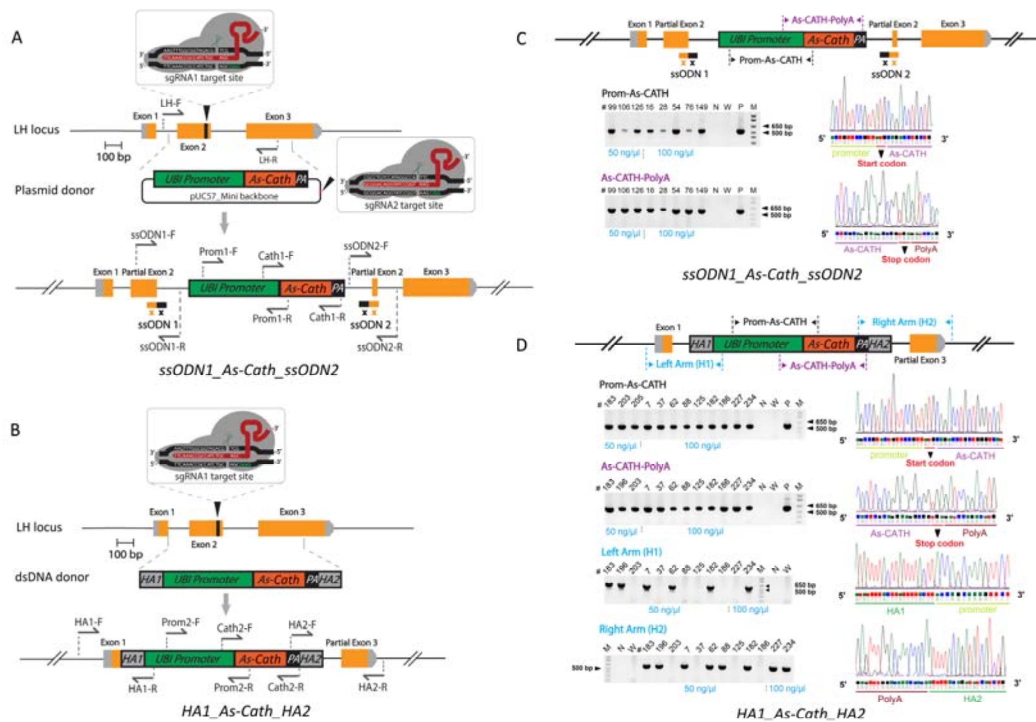

Fig. 1. Single-stranded oligodeoxynucleotide (ssODN) and linear double-stranded DNA (dsDNA) with CRISPR/Cas9 mediating knock-in (KI) at the *luteinizing hormone* (*LH*) locus of channel catfish. (A) Schematic illustration of the insert-specific region for the cathelicidin gene from *Alligator sinensis* (*As-Cath*) KI via the two-hit two-oligo (2H2OP) system assisted by ssODNs at the *LH* locus, named 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* gene using PCR amplifications. **(B)** Schematic diagram of the *As-Cath* KI via the dsDNA system, named HA1_As-Cath_HA2 donor. Primers show the strategy to test the HA junctions, UBI promoter

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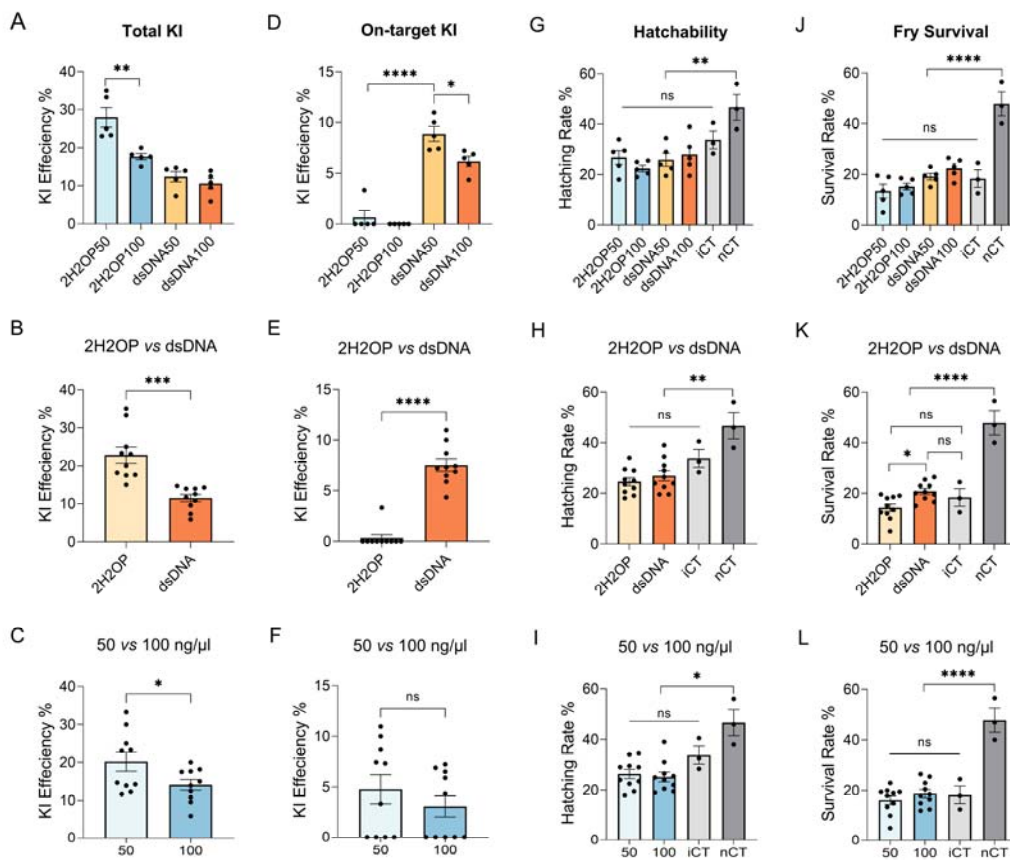
853 region, and *As-Cath* gene region. **(C)** TAE agarose gel of PCR amplicons showing off-target
854 positive detection of the ssODN1_As-Cath_ssODN2 construct using 2H2OP method. The
855 promoter region (Prom-As-CATH, 519 bp) and *As-Cath* region (As-CATH-PolyA, 591 bp)
856 were illustrated with sequencing results. **(D)** TAE agarose gel of PCR amplicons showing
857 on-target positive detection of the HA1_As-Cath_HA2 construct using dsDNA method. The
858 targeted gene regions (Prom-As-CATH, 542 bp and As-CATH-PolyA, 597 bp) and the
859 junctional regions (HA1, 573 bp and HA2, 598 bp) were determined with sequencing results.
860 The numbers on the top of the gel images indicate the sample IDs of the fish. Lane N,
861 negative control using water as template; Lane W, wild-type control (nCT); Lane P, positive
862 (plasmid or dsDNA donor) control; Lane M, DNA marker (1 kb), 500 and 650-bp bands are
863 highlighted with black triangles; 50 and 100 ng/μL show the different doses of donors:
864 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.

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875 **(K, L)** Comparison of the fry survival rate for different systems or dosages. iCT,
876 sham-injected control; nCT, non-injected control; 2H2OP(50/100), the
877 CRISPR/Ca9-medicated system with ssODN1_As-Cath_ssODN2 construct (with a
878 pUC57_mini plasmid and ssODN donor as 50/100 ng/μL); dsDNA(50/100), the
879 CRISPR/Ca9-medicated system with HA1_As-Cath_HA2 donor DNA (with a dsDNA donor
880 as 50/100 ng/μL); * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$; ns = not
881 significant, by unpaired student's *t*-test or one-way ANOVA.

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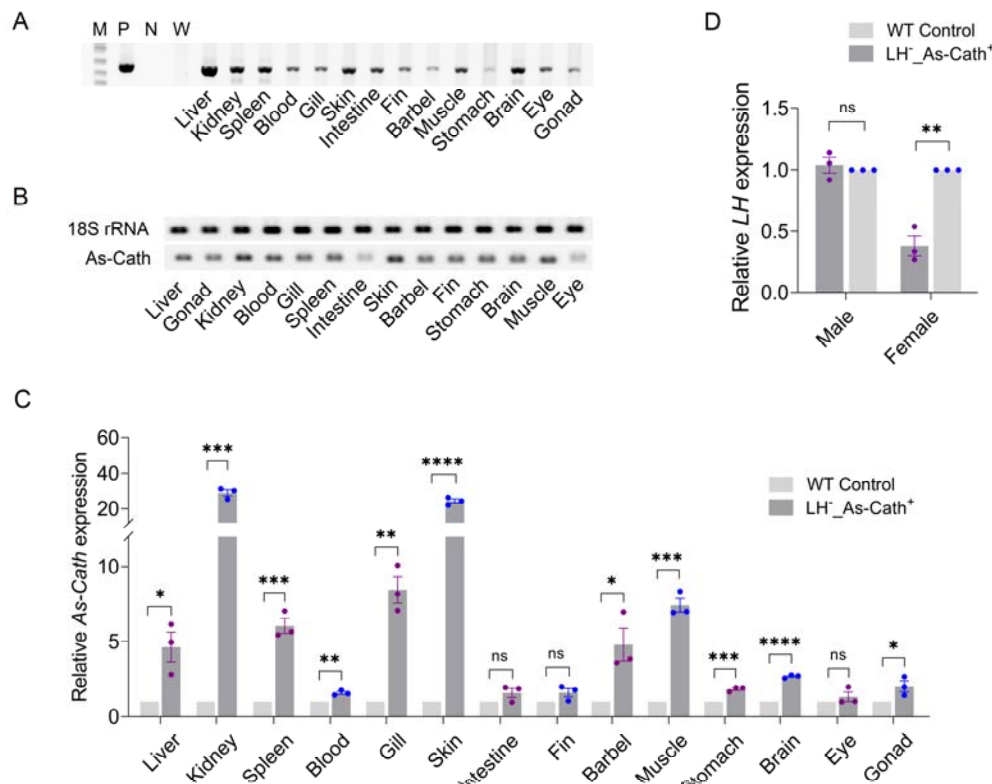

885 **Fig. 3. Mosaicism detection and the expression of the cathelicidin gene from *Alligator***
886 ***sinensis* (*As-Cath*) in the LH⁻_As-Cath⁺ fish line. (A)** PCR amplicons show the *As-Cath*
887 region in 14 tissues from one representative LH⁻_As-Cath⁺ fish. **(B)** The agarose gel
888 electrophoresis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

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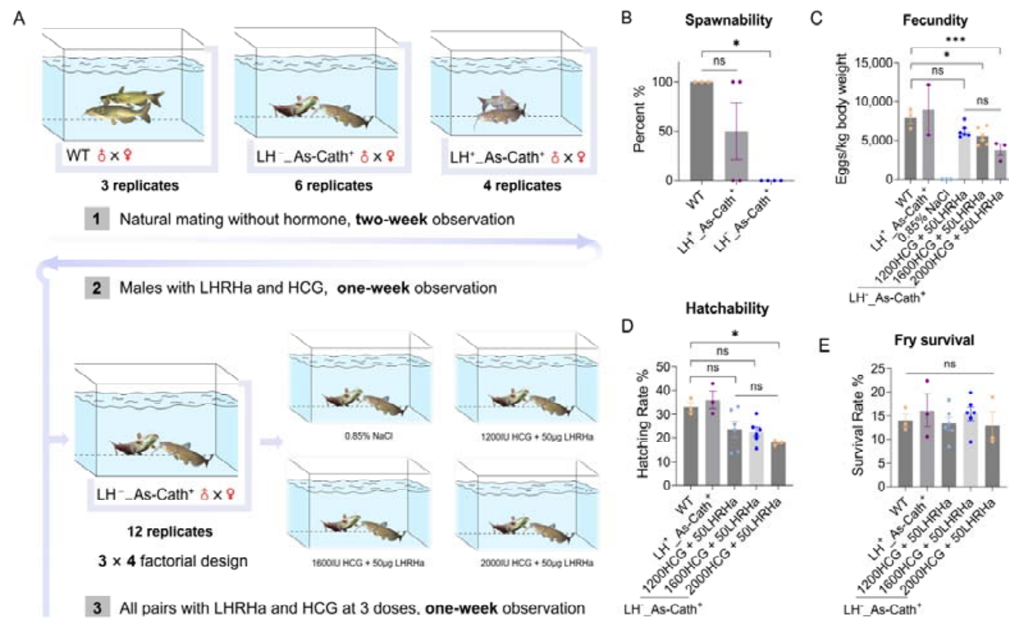

Fig. 4. Reproductive determination and restoration of the As-Cath-integrated fish lines. **(A)** A three-round design of the reproduction experiment. Three genotypes of P_0 founders: WT, LH⁻_As-Cath⁺, and LH⁺_As-Cath⁺ fish were involved. First round, 3, 6 and 4 pairs as replicates for each genotype were 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⁻_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 doses of LHRHa and HCG in a 3 × 4 factorial design for one week. **(B)** Detection of spawnability for LH⁻_As-Cath⁺ fish during natural mating. **(C, D, E)** Potential effects of different hormone treatments on the fecundity and hatchability of P_0 generation, and fry survival of F_1 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.

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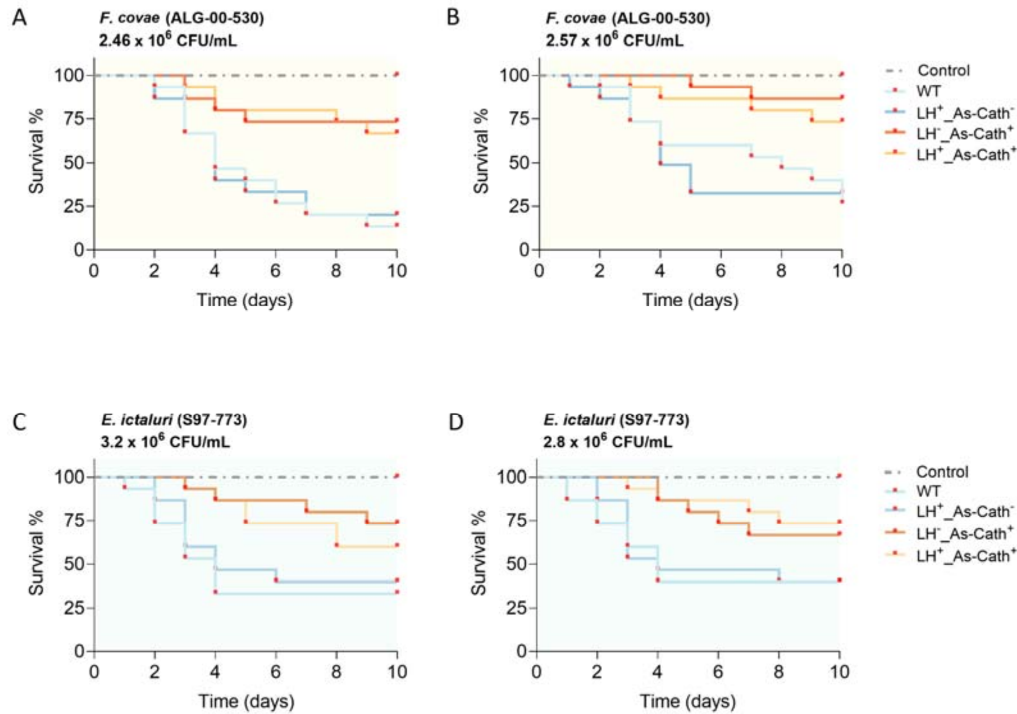

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924 **Fig. 5. Kaplan-Meier plots of *As-Cath* integrated catfish against two fish bacterial**
925 **pathogens. (A, B)** Survival curves of P_0 and F_1 generations for a variety of genotypes
926 infected by *Flavobacterium covae*, respectively. **(C, D)** Survival curves of P_0 founders and F_1
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^+_As-$Cath^-$, negative fish line (micro-injected fish
932 without *LH* mutation and *As-Cath* insertion); LH^-_As-$Cath^+$, on-target positive fish (*As-Cath*
933 insertion was detected at *LH* locus); LH^+_As-$Cath^+$, off-target positive fish (*As-Cath* insertion
934 was detected but not at *LH* locus).

935 **Table 1.** Target sequences of sgRNAs and the universal primer used in the present study. Underlined sequences represent the protospacer
936 adjacent motif.
937

sgRNA	Targeted sequence for sgRNA (5'-3')
sgRNA1	5'- TTCAAACCGCCATCTGCAGCGGG -3'
sgRNA2	5'- GCGGACAGGTATCCGGTAAGCGG -3'
Universal primer	TTTTGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAG CCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

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Table 2. Mean monthly body weight (BW), sample size (N) over time of P_0 and F_1 As-Cath-integrated, negative and control channel catfish, *Ictalurus punctatus*. P_0 founders were generated in June 2020, and F_1 progeny were produced in June 2022. For both generations, four genotypes: WT, LH^{+}_As-Cath^{-}, LH^{-}_As-Cath^{+}, and LH^{+}_As-Cath^{+} 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 ± 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
P_0	WT	27.20 ± 1.77^{a}	60	37.15 ± 2.83^{a}	30	42.45 ± 3.08^{ab}	30	36.75 ± 2.31^{a}	30	50.75 ± 3.58^{a}	27
	LH^{+}_As-Cath^{-}	26.30 ± 2.24^{a}	60	36.40 ± 2.14^{a}	30	38.30 ± 3.20^{a}	29	35.25 ± 3.18^{a}	29	51.10 ± 2.28^{a}	29
	LH^{-}_As-Cath^{+}	23.10 ± 1.72^{a}	41	41.30 ± 2.60^{a}	28	49.65 ± 2.35^{b}	21	43.20 ± 2.75^{a}	20	58.45 ± 4.21^{a}	20
	LH^{+}_As-Cath^{+}	27.75 ± 2.39^{a}	63	39.95 ± 2.73^{a}	32	47.25 ± 3.26^{ab}	33	34.50 ± 3.58^{a}	33	50.85 ± 2.89^{a}	33
		8/9/2022		9/11/2022		10/12/2022					
		BW	N	BW	N	BW	N				
F_1	WT	2.63 ± 0.16^{a}	60	15.13 ± 1.00^{a}	54	22.90 ± 1.23^{a}	54				
	LH^{+}_As-Cath^{-}	2.60 ± 0.16^{a}	60	14.67 ± 0.91^{a}	56	21.30 ± 1.03^{a}	54				
	LH^{-}_As-Cath^{+}	3.03 ± 0.14^{a}	60	19.57 ± 1.31^{b}	59	26.03 ± 1.32^{b}	57				
	LH^{+}_As-Cath^{+}	2.70 ± 0.12^{a}	60	13.14 ± 1.05^{a}	58	22.13 ± 1.09^{a}	58				

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

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