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Genome Manipulation Advances in Selected Aquaculture Organisms

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ABSTRACT

With the rising global demand for seafood and the challenges posed by overfishing and climate change, the aquaculture sector has become increasingly important in providing high-quality protein for human consumption. Although traditional selection breeding programs have made great strides in genetic improvement of aquaculture species over the past decades, faster and more precise breeding tools, such as genome manipulation, are needed for performance enhancement of aquaculture stock. This review presents a comprehensive overview of the current status of three major genome manipulation tools, including RNA interference (RNAi), gene transfer, and genome editing in aquaculture species, and discusses the advances made, challenges faced, and potential future directions of this fast-developing field. Taking catfish as an example, this paper reviews the specific applications of these techniques to improve traits such as growth, disease resistance, reproduction, and nutritional profiles in various commercially important fishes and crustaceans, highlighting successful applications and ongoing research efforts. We also propose CRISPR/Cas9-mediated multiplex genome editing for the knockout or replacement of multiple genes in parallel to improve multiple traits in fish. Collectively, this review provides insights into the evolving landscape of genome manipulation in aquaculture and sheds light on its implications for sustainable practices and responsible innovation.

1 | Introduction

The global human population could grow to 9.7 billion by 2050, and it is projected to peak at around 10.4 billion people in the 2080s and to remain at that level until 2100 [1]. It has been a challenge to feed the expanding population due to climate change, limited arable land/water availability, and other issues. In addition to crops and livestock, fish are indispensable sources of protein for humans, especially in low- and middle-income countries [2]. However, this high demand for fish

protein has put a significant strain on wild fish populations because of near- or above-sustainable harvest exploitation. Aquaculture promises to fill this gap as a fast-growing contributor to the world's food supply, with global consumption expected to reach 25.5 kg per capita by 2050 [3]. Nevertheless, improving the productivity and sustainability of aquaculture is essential for achieving future global food security. Scientific breakthroughs and technological innovations in aquaculture are urgently needed to relieve the pressure on food systems globally.

Abbreviations: AMP, antimicrobial peptide; dsDNA, double-stranded DNA; dsRNA, double-stranded RNA; GH, growth hormone; GS, genomic selection; GWAS, genome-wide association study; KD, knockdown; KI, knock-in; KO, knockout; MAS, marker-assisted selection; RNAi, RNA interference; shRNA, short-hairpin RNA.

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Genetic variation is the basis of performance enhancement [4], and selective breeding aims to create and exploit genetic variation. Traditional approaches to improving production traits in modern aquaculture, including intraspecific crossbreeding, interspecific hybridization, and selection, can enhance economically important traits, and have been applied to various aquaculture species. For instance, farmed fishes that have been genetically improved for survival, feed conversion rate, reproductive performance, carcass yield, and disease resistance through traditional selection and breeding include channel catfish (*Ictalurus punctatus*) [5–8], Atlantic salmon (*Salmo salar*) [9, 10], rainbow trout (*Oncorhynchus mykiss*) [11], and Nile tilapia (*Oreochromis niloticus*) [12], among others. Crossbreeding refers to the process of mating individuals from two different breeds, strains, or lines to produce offspring with desired traits improved by heterobeltiosis [13] (Figure 1A). For example, in channel catfish, intraspecific crosses of two strains (Marion and Kansas) improved not only growth, but also resistance to enteric septicemia of catfish (ESC), columnaris disease, and parasites [14] and increased tolerance to low dissolved oxygen (DO) [15]. Interspecific hybridization is the breeding or mating between individuals of two species to obtain progeny with desired traits [4]; backcrossing is advantageous in some cases to reduce labor intensity [16]. The best type of catfish for pond aquaculture in North America is the interspecific hybrid, channel catfish ♀ × blue catfish (*I. furcatus*) ♂ (CB hybrid), which showed multiple enhanced traits, including growth rate, dressout and fillet percentage, disease resistance, harvestability and tolerance to low DO compared to their pure-species parents [13, 17, 18].

Genomic studies reveal the architecture and genome complexity of aquatic species, which in turn benefits the understanding of gene functions and provides the molecular basis for marker-assisted selection (MAS) and genomic selection (GS). MAS is a quantitative breeding technique that uses molecular markers to aid selection for desirable traits [19]. Several steps are involved for MAS in aquaculture: (1) Identify specific genes or genetic markers that are associated with desirable traits, such as fast growth, high disease resistance, or better adaptability to environmental conditions; (2) assess the genotype within families in the breeding population by analyzing marker-trait associations; (3) select individuals with desired marker alleles for breeding; (4) maintain a breeding program integrating MAS, continually validating marker-trait linkages, and (5) refine breeding approaches to enhance genetically determined traits.

A genome-wide association study (GWAS) scans the entire genome of individuals within a population to pinpoint specific genetic variations associated with phenotypic traits of interest. In practice, these approaches can also be complementary, with GWAS informing the discovery of new markers for MAS (Figure 1B). In aquaculture, quantitative trait locus (QTL) or economic trait locus (ETL) identified by QTL detection or GWAS may be used in MAS [13, 19]. An increasing number of QTLs and single-nucleotide polymorphisms (SNPs) for growth, disease resistance, and hypoxic tolerance have been identified in aquaculture species [20–22], and several studies have implanted MAS [23]. Three successful examples of application of MAS for

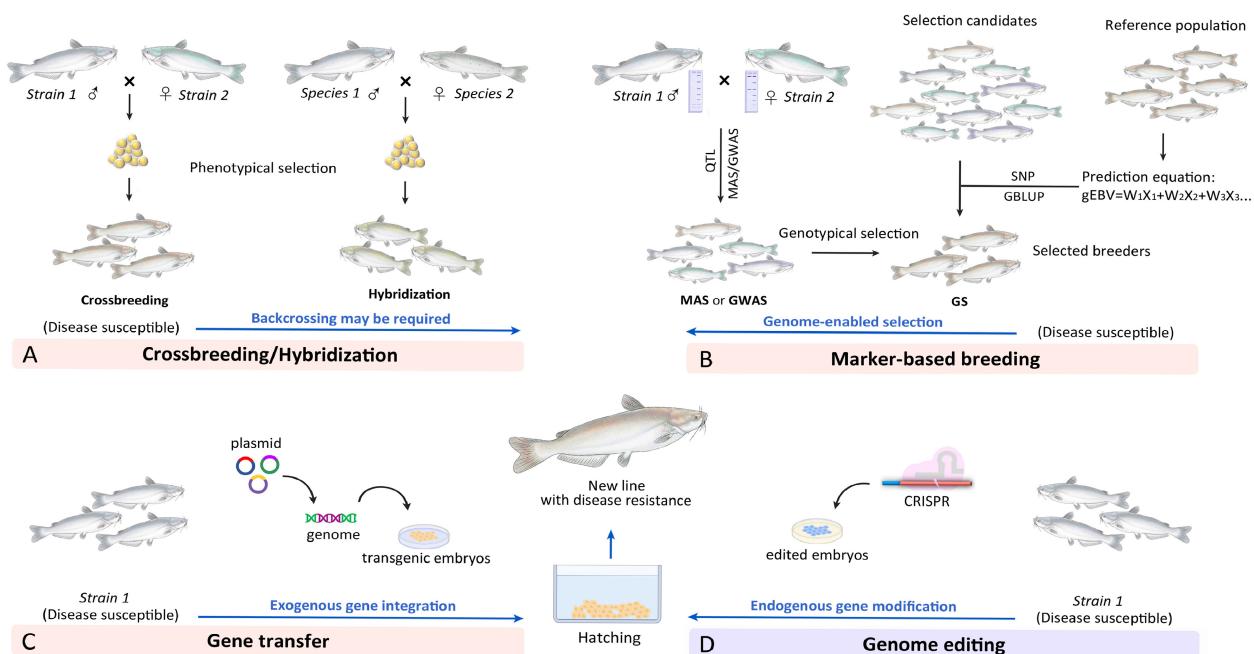


FIGURE 1 | Representative breeding approaches that are commonly used in modern aquaculture. Crossbreeding refers to intraspecific crossing, and the process of mating individuals from two different breeds, strains, or lines to produce offspring with desired traits (e.g., disease resistance) with immediate improvement through heterosis. Hybridization refers to interspecific hybridization. The breeding or mating between individuals of two different species (e.g., channel catfish ♀ (*Ictalurus punctatus*) × blue catfish ♂ (*I. furcatus*)) to obtain progeny with desired traits. Marker-based breeding refers to a modern technique (e.g., marker-assisted selection and genomic selection) used in plant and animal breeding that leverages molecular markers to select desirable traits more efficiently than traditional breeding methods. Gene transfer, the transfer of a specific gene or piece of DNA from one species to another (or the same species) using recombinant DNA technology. Genome editing: A type of genome manipulation that enables precise modification of an organism's genome. CRISPR, clustered regularly interspaced short palindromic repeats; QTL, quantitative trait locus; SNP, single nucleotide polymorphism; GWAS, genome-wide association study; GBLUP, genomic best linear unbiased prediction.

enhancement of disease resistance in aquaculture species are Japanese flounder (*Paralichthys olivaceus*) [24], Atlantic salmon [25], and rainbow trout [26].

Compared to MAS, GS is a genome-based breeding tool that utilizes the entire genome of a species to predict its breeding value for specific traits based on GS models (e.g., genomic best linear unbiased prediction and Bayesian) (Figure 1B), and requires larger datasets and more complex genomic analyses to provide more accurate selection [19, 27]. Thus, many theoretical papers have been written, but few responses to GS-based breeding experiments have been conducted in fishes. Most GS-related studies have focused on growth (body weight and length) and disease resistance in salmonids using various SNP markers, including Atlantic salmon, rainbow trout and coho salmon (*O. kisutch*) [19]. GS also has been applied to mollusks and crustaceans, such as Zhikong scallop (*Chlamys farreri*) for growth [28], Pacific oyster (*C. gigas*) for disease resistance [29], Pacific white shrimp (*Litopenaeus vannamei*) for disease resistance [30], and banana shrimp (*Fenneropenaeus merguiensis*) for growth and disease resistance [31]. Recently, a genotype of large yellow croaker (*Larimichthys crocea*) was established using GS models, and selected broodstock showed increased resistance to the parasite *Cryptocaryon irritans*, as reflected by a 50% increase in survival rate [32]. Nevertheless, several shortcomings limit popularization and application of GS in aquaculture. For instance, insufficient diversity of the training population [33] and genotype-environment interactions [34] compromise the predictive accuracy of GS.

Looking beyond these traditional and more-recent genomic-selection approaches, the advancement of aquaculture breeding programs can benefit from the application of RNA interference (RNAi), gene transfer (transgenesis) and genome editing (Figure 1C,D). Here, we address each of these genomic manipulation techniques below. As the longest used technique, RNAi has been applied to prawns/shrimps, crabs, and shellfish to enhance performance and identify functional genes, especially for improved disease resistance and reproduction control [40]. Regarding transgenesis, a variety of growth hormone (GH) genes have been transferred into the genomes of farmed fishes to improve production efficiency since the mid-1980s when the first transgenic fish (goldfish, *Carassius auratus*) were produced expressing an introduced growth hormone transgene [35]. Subsequently, the transfer of antimicrobial peptide (AMP) genes or antifreeze polypeptide/protein (AFP) genes conferred heightened disease resistance or elevated tolerance to low temperatures in fishes [36, 37]. In recent years, gene transfer and genome-editing tools have been used to greatly improve economic traits in fishes. Successful examples of such food fishes that have been developed by research institutions include GH transgenic Atlantic salmon [38, 39], leptin receptor (*lepr*) gene-edited tiger puffer (*Takifugu rubripes*), and myostatin knockout red sea bream (*Pagrus major*) [40]. However, gene transfer and genome editing are rarely applied to crustaceans for commercial trait improvement due to their having small embryos with hard shells and the release of late-stage embryos from the mother [4].

New developments of genome manipulation tools, including RNAi, gene transfer and genome editing, have been applied to

aquaculture to create heritable variations at the DNA or RNA level, allowing dramatic increases in the production or performance of fishes and crustaceans. In this review, we describe the current status of genome manipulation with an emphasis on the gene silencing, transgenesis, and targeted gene modification/replacement that can be produced using these three techniques, as well as the application of multiplex genome editing as a next-generation breeding technology for aquaculture improvement.

2 | Main Genome Manipulation Tools

Traditional selection breeding approaches have been applied in aquaculture breeding programs with great success by exploiting genetic variation. However, large portions of the genomes of major aquatic species are fixed, and genetic variability maybe limited [41], limiting the potential to improve many traits. In this context, genome manipulation tools beyond these conventional approaches provide promising alternatives to enhance traits in economically important fish species, and offer direct and innovative solutions for sustainable aquaculture development.

2.1 | RNAi

RNAi, is a naturally occurring biological process that regulates gene expression by silencing specific target genes through the introduction of complementary RNA molecules [42], and occurs in eukaryotic cells ranging from plants to animals, including human beings [43]. The process involves the expression of small RNA molecules, including small interfering RNA (siRNA) or microRNA (miRNA), to trigger the degradation or inhibition of expression of complementary mRNA, ultimately terminating the translation of the corresponding proteins. RNAi begins with the production of either of these two small RNA molecules, which can be generated from either exogenous or endogenous sources of double-stranded RNA (dsRNA), such as plasmid-containing primary miRNA (pri-miRNA) or short hairpin RNA (shRNA) [44]. Viral RNA or artificially introduced dsRNA as exogenous molecules are usually processed by the Dicer enzyme into smaller fragments (siRNAs or miRNAs). They are then loaded into the multi-protein RNA-induced silencing complex (RISC), which is guided by the Argonaute protein for target mRNA cleavage (if it has perfect complementary) or translational repression (if it has imperfect complementary) [42, 44] (Figure 2A).

The RNAi phenomenon was first reported in pigmented petunia petals by Napoli et al. [45], followed by the first RNAi study in an animal, the nematode (*Caenorhabditis elegans*), using sense or antisense RNA to degrade the *par-1* message [46]. One of the most significant breakthroughs facilitated by RNAi is the application of dsRNA. Fire et al. [47] found that dsRNA induced a more potent sequence-specific silencing response than the commonly used single-stranded antisense RNA in *C. elegans*, establishing a new conceptual framework for RNAi-mediated gene silencing by highlighting a role for dsRNA (Figure 3). To date, RNAi as a novel genome manipulation tool has been widely applied in the life sciences, and was awarded the 2006 Nobel Prize to Andrew Fire and Craig Mello in Physiology or Medicine, and RNAi-based drugs, such as lumasiran, have been approved by governments [48, 49].

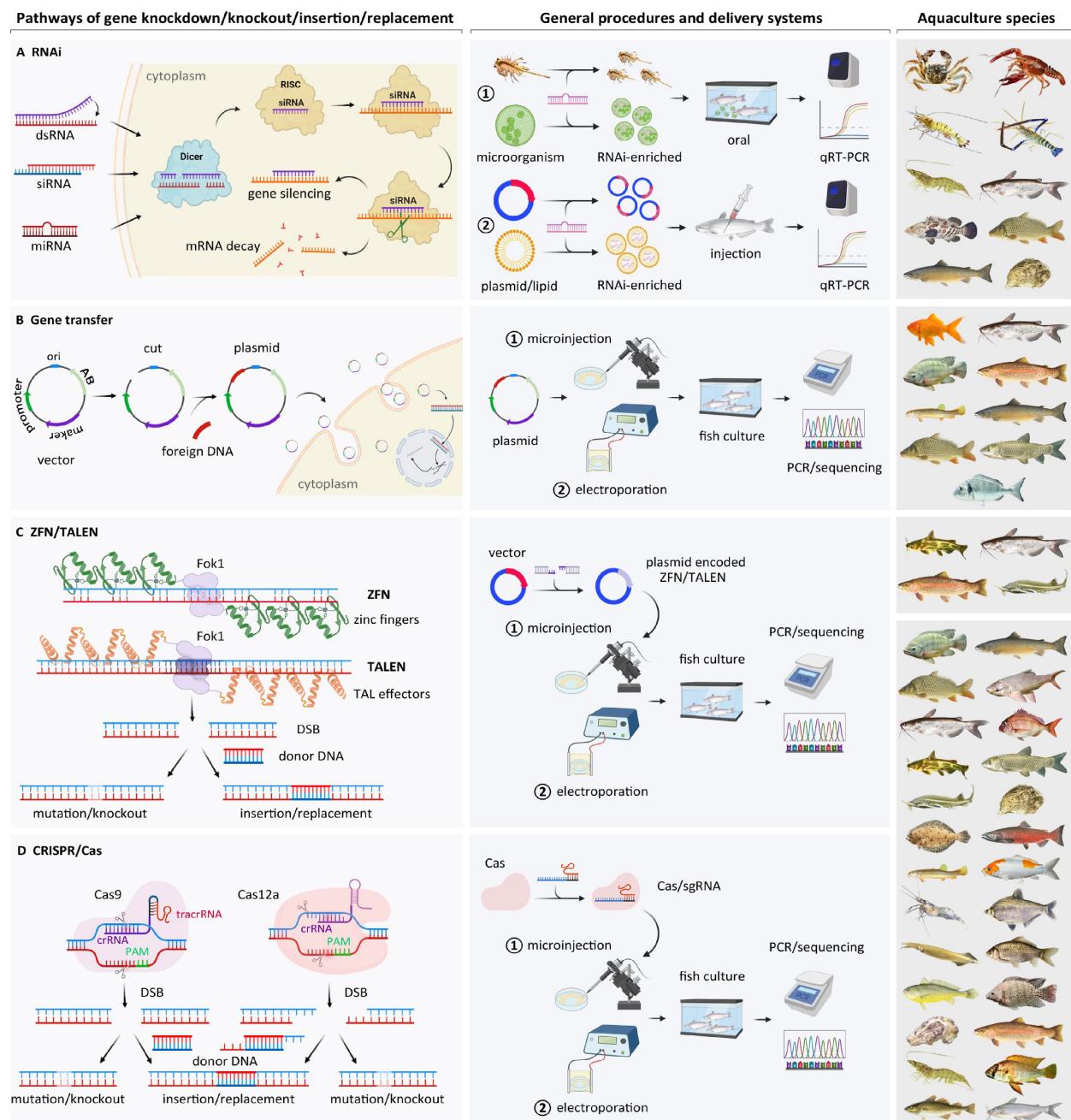


FIGURE 2 | Applications of major genome manipulation tools in aquaculture species. Gene knockdown/knockout/replacement pathways, general procedures and delivery systems and main aquaculture species are presented. (A) Schematic illustration of the RNA interference (RNAi) pathway using different RNAi molecules. Injection of naked/engineered RNAi molecules and oral administration of microorganism-enriched dsRNA are mainly methods used for delivery. (B) Traditional gene transfer using recombinant DNA. Recombinant plasmids can typically be delivered into embryos by microinjection or electroporation. (C) Genetic modifications generated by ZFN-/TALEN-mediated genome editing. (D) Comparison of CRISPR/Cas9- and CRISPR/Cas12a-mediated genome editing. Microinjection and electroporation are main delivery methods for genome editing in aquaculture species. dsRNA, double-stranded RNA; siRNA, small interfering RNA; miRNA, microRNA; RISC, RNA-induced silencing complex; ZFN, Zinc-finger nuclease; TALEN, transcription activator-like effector nuclease; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9/12a, CRISPR-associated protein 9/12a; DSB, double-stranded break; PAM, protospacer adjacent motif.

The delivery of RNAi molecules to aquatic animals poses unique challenges because of extra- and intra-cellular biological barriers and the aqueous environment. Various methods have been explored for delivering RNAi molecules into crustaceans, mollusks or fishes, both *in vivo* and *in vitro* (Figure 2A). In some species, including Atlantic salmon, immersing individuals in a solution containing RNAi molecules

is a simple and non-invasive method, [43]. RNAi molecules can be added directly to the water, allowing the research subject to absorb the molecules through their gills or other external surfaces. However, delivering RNAi via immersion faces challenges like uneven distribution, rapid degradation, and dosage control [43]. The most common approach is injection, which provides precise delivery with high efficiency, but

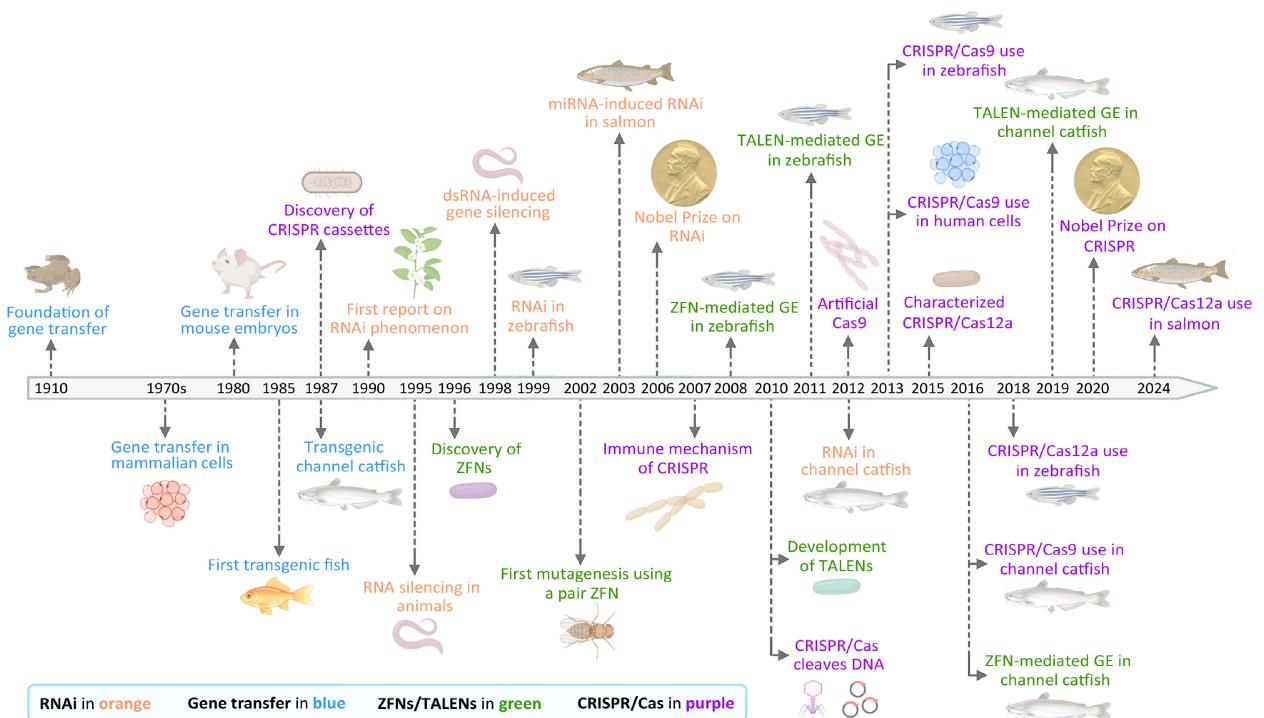


FIGURE 3 | Key events in the discovery and application of genome manipulation tools in aquaculture. The timeline shows the year of related publications, and the species involved in the experiment are presented. GE, genome editing. RNAi, RNA interference; ZFN, Zinc-finger nuclease; TALEN, transcription activator-like effector nuclease; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9/12a, CRISPR-associated protein 9/12a; dsRNA, double-stranded RNA; miRNA, microRNA.

potentially causes stress to the animals. Invertebrates, like crustaceans, can receive RNAi through hemolymph injections. Alternatively, incorporating RNAi molecules into the diet of aquatic animals is a practical method for species with oral intake capabilities. This approach can involve admixing RNAi molecules into the feed or incorporating them into specific feed formulations. For instance, dsRNA-enriched bacteria can be fed to giant tiger prawn (*Penaeus monodon*) using *Artemia* as a delivery system, leading to increased cumulative survival rates and delayed average time-to-death following the Laem-Singh virus (LSNV) challenge [50]. Electroporation also can be adapted to deliver RNAi molecules into fish embryos. Su et al. [51] and Li et al. [52] successfully electroporated dsRNA- or shRNA-carrying plasmid to knock down (KD) expression of the *nanos* and *dead end* (*dnd*) genes in channel catfish. RNAi molecules encapsulated into lipid nanoparticles offer an additional option for injection or other delivery methods to improve KD efficiency. Sarathi et al. [53] suggested that feeding giant tiger prawn with chitosan complex nanoparticle-coated dsRNA can inhibit *vp28* gene expression of white spot syndrome virus (WSSV) during infection. To reduce RNA degradation, cationic polysaccharide chitosan-wrapped dsRNA nanoparticles (chitosan-dsRNA nanoparticles) were injected into Pacific white shrimp (*L. vannamei*) to KD the *Rab7* gene with high efficiency [54]. Recently, several off-the-shelf commercial reagents, such as Lipofectamine 2000 and Oligofectamine (Invitrogen), are being routinely employed to transfect siRNA into fish cell lines, including common carp (*Cyprinus carpio*) [55], Chinook salmon (*O. tshawytscha*) [56], and grass carp (*Ctenopharyngodon idella*) line [57]. Oral administration of dsRNA-enriched *Artemia* or microorganisms

is more promising due to the high yield of RNAi molecules compared to injection or immersion of naked dsRNA in practice [43], but more effective approaches for large-scale applications need to be developed.

While RNA interference (RNAi) has been widely studied in cell lines, its applications for in-vivo trait improvement are limited. This is primarily due to challenges in achieving effective and consistent RNAi delivery to target tissues, as well as issues with RNAi stability and degradation in complex living systems. In aquaculture, RNAi-mediated strategies hold significant potential to reduce infectious diseases, improve growth rates, and control reproduction. Wargelius et al. [58] published the first report on the application of RNAi in a fish species, zebrafish (*Danio rerio*), using dsRNA, and a higher efficiency was observed compared to antisense RNA-mediated RNAi. Subsequently, small interfering RNAs (siRNAs) were used to KD the green fluorescent protein (*GFP*) and tyrosinase A (*tyrA*) genes with low off-target effects (e.g., unintended gene expression) on the expression of unrelated genes in an economic species, rainbow trout [59]. Such studies are evidence that RNAi is applicable in fish species. At that time, most studies focused on gene transfer in fish species, whereas RNAi was widely used in crustaceans to improve performance and identify functional genes, especially for enhancing disease resistance. By selectively suppressing viral gene expression, RNAi-based strategies have shown promising results in controlling viral diseases, such as WSSV [60, 61], grass carp reovirus (GCRV) [57], viral hemorrhagic septicemia virus (VHSV) [56, 62], and rock bream iridovirus (RBIV) [63]. In addition, reproductive control and sex reversal of several species,

such as channel catfish, common carp, Chinese mitten crab (*Eriocheir sinensis*), and giant river prawn (*Macrobrachium rosenbergii*) were induced by RNAi-mediated gene silencing [64–67]. Recently, studies focusing on growth improvement were conducted upon Nile tilapia [68, 69] and red abalone (*Haliotis rufescens*) [70] by knocking down the myostatin (*mstn*) or steroidogenic factor 1 (*sf1*) genes (Table S1).

Despite its potential, the practical implementation of RNAi in aquaculture faces challenges such as incomplete gene silencing, development of efficient delivery strategies, and off-target effects. The silencing efficiency of target genes is not 100% in some cases, resulting in incomplete elimination of gene expression, which allows normal gene and protein function [71, 72]. Another drawback is that the injection of RNAi molecules is time-consuming and labor-intensive to apply on a large scale in aquatic species, even if it enables high efficiency. In addition, RNAi can inadvertently KD multiple non-targeted genes, resulting in off-target effects, with undesirable trait outcomes [73]. To overcome these hurdles and pave the way for widespread adoption of RNAi as a transformative tool in aquaculture, ongoing research and technological advances will have to continue.

2.2 | Gene Transfer

Gene transfer uses a vector as a delivery vehicle which can result in integration of the introduced gene into the host genome and expression of the introduced gene (Figure 2B). This technique is widely used in genome manipulation to modify aquaculture species for various purposes, such as studying gene expression/function, producing therapeutic proteins, or improving production traits [4]. The key to transgenesis success is the fusion of foreign genes to a suitable promoter sequence in a functional expression vector leading to stable gene expression, although transgenes may show no/low expression levels if the recombinant DNA does not include a suitable promoter. In practice, the transgenesis of interest is integrated into an artificial promoter-driven expression vector to circumvent regulatory mechanisms that inhibit gene expression. Various viral promoters, such as simian virus type 40 (V40), Rous sarcoma virus (RSV), and cytomegalovirus (CMV), have been identified and used in fish transgenesis, leading to high gene expression in the host species, including Nile tilapia, goldfish and channel catfish [74–76]. Limited research on viral promoters is currently being conducted due to the relatively poor control over transgene expression in host tissues and to poor public perception [4]. Recently, “all-fish” promoters, including β -actin from common carp and ubiquitin (UBI) from zebrafish, have been applied in transgenic catfish species [77–81], increasing the selection of available promoters.

Common approaches to transgenesis, including virus-based transfection, electroporation, microinjection, or gene-gun bombardment, can be used to deliver recombinant DNA/donor constructs into host cells [4]. Microinjection uses a fine glass needle to inject substances directly into cells or embryos, requiring a micromanipulator and microscope in a clean, sterile laboratory environment. Electroporation employs electric fields to temporarily open cell membranes for the uptake of molecules like DNA, using an electroporator and specialized electrodes, typically in a lab equipped for cell culture. Further, sperm- and

transposon-mediated gene transfer were developed in model fishes [82, 83]. Microinjection was used initially for the introduction of genes into early-stage embryos in model and aquaculture species. However, microinjection is time-consuming and labor-intensive, coupled with a high mortality rate of eggs and embryos [84, 85]. For example, microinjection resulted in mortality ranging from 57% to 84% in channel catfish [77, 84, 86], 63% to 78% in common carp [87, 88], and 64% in rainbow trout [89]. In comparison to channel catfish, the embryos of white catfish (*Ameiurus catus*) and blue catfish are more sensitive to microinjection-induced physical damage, as reflected by low survival (~5%) [79]. In this case, electroporation is more suitable to minimize oocyte lethality. Some studies have shown that electroporation leads to higher integration rates (sometimes as much as 30%–100%) than microinjection [85]. However, in some cases, electroporation results in lower or similar integration rates compared to microinjection, even in the same fish species. An integration rate of 16% or 19% was observed when the donor plasmid was microinjected into channel catfish embryos at various concentrations, whereas this efficiency was 9.5% or 17.6% when electroporation was applied [77]. This variation in gene integration efficiency may result from the genetic background of the family/strain, target locus and foreign gene rather than from the method of delivery itself [4]. Electroporation has consistently shown considerable insertion efficiencies with low mortality for mass egg-based transgenesis. However, it also has limitations, such as the need for a large amount of donor constructs. Indeed, microinjection is the most frequently used method in aquaculture species because of its convenience and low cost [90].

As an older tool of genome manipulation, the foundation for gene transfer was actually laid as early as 1910, when the cell constituents were transferred into frog eggs [91] (Figure 3). By the early 1970s, the potential of gene transfer technology for understanding gene expression and altering phenotypes became evident [92]. Advances in gene cloning, coupled with microinjection procedures for amphibian eggs, fueled the rapid expansion of gene-transfer studies. During the 1970s and 1980s, an increasing number of studies focused on transgenesis were conducted in cultured mammalian cells and mouse embryos [93–97], laying the groundwork for similar research in fish. Compared to RNAi, transgenesis in aquaculture focused on the improvement of growth and disease resistance traits in fish species. The first transgenic goldfish carrying the human GH gene was produced by Zhu et al. [35], making the beginning of a significant rise in transgenesis in aquaculture research. Early breakthroughs by Chourrout et al. [98] and Dunham et al. [84] led to the creation of transgenic rainbow trout and channel catfish with the GH gene, setting the stage for further developments in the field. Over the next two decades, more than a dozen species were engineered to carry the GH gene to enhance growth [90] (Table S2). In parallel, transgenesis was used to introduce AMP or AFP genes to improve resistance to bacteria, parasites and viruses or to increase tolerance to low temperatures [4]. Despite these advances, during the first three decades of transgenesis research, foreign genes were integrated randomly into the host genome, resulting in mosaic patterns of transgene insertion into the host genome and expression [4]. Traditional transgenesis is gradually being replaced by more advanced systems, such as the use of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-mediated genome-editing

platform, which allows for gene knockout or knock-in (KI) of transgenes of the target genome with high efficiency.

2.3 | Genome Editing

Genome editing encompasses a range of tools that allow precise modification of targeted DNA sequences. Prominent among these are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9. These genome-editing tools use a variety of endonucleases to induce double-strand breaks (DSBs) of DNA, followed by innate DNA repair in cells, to facilitate targeted alterations to the genome. ZFNs have separate DNA-binding (a pair of three ZFNs) and DNA-cleavage (*FokI*, a restriction enzyme) domains [99]. The zinc fingers bind to target locations on the genome and fuse to the *FokI* to cleave specific DNA sequences. TALENs followed in the footsteps of ZFNs, igniting the genome-editing revolution, and are made by fusing a transcription activator-like (TAL) effector DNA-binding domain (a pair of six TALEs) to a DNA-cleavage domain (*FokI*) [100]. CRISPR is a family of DNA sequences found in the genomes of prokaryotic organisms, such as bacteria and archaea [101], and plays a key role in the bacterial defense system by providing a form of acquired immunity [102, 103]. The CRISPR/Cas9 system consists of two components: the single-guide RNA (sgRNA) and Cas9 protein. The sgRNA directs Cas9 to specific DNA sequences, where it induces DSBs [104]. CRISPR/Cas9-assisted genome editing can be performed by co-injection of sgRNAs coupled to mRNA encoding Cas9 protein or by injection of the ribonucleoprotein (RNP) complexes made of Cas9 protein and sgRNAs. Two major DNA repair mechanisms, homologous recombination (HDR) and non-homologous end joining (NHEJ), are involved when DSBs are induced, regardless of the genome-editing tool employed. Small insertions and deletions (indels) for gene knock-out (KO) are induced by the NHEJ pathway in the absence of a donor DNA. In contrast, gene replacements/KIs are predominantly induced by the HDR pathway when a donor DNA template is provided (Figure 2C,D).

With respect to aquaculture, genome editing allows precise DNA modifications, requiring procedures like microinjection or electroporation systems to deliver CRISPR components, along with PCR machines and gel electrophoresis for molecular analysis. High-resolution microscopes are essential for embryo manipulation in some cases. In laboratory working with fishes, specialized microinjection stations and electroporators are needed as well, along with well-maintained aquaculture facilities featuring temperature-controlled recirculating water systems, breeding tanks, and appropriate lighting. These setups support precise genome editing, successful development of genetically modified fish, and reliable experimental outcomes.

Although microinjection and electroporation are two main approaches for the delivery of genome-editing reagents, nanoparticle-mediated delivery of CRISPR/Cas9 via endocytosis, such as lipid-, nanotube-, and nanogold-encapsulated CRISPR/Cas9-sgRNA complexes, have been developed in mammalian cells [105]. This advanced method produces on-target mutations/modifications with high efficiency and low mortality in vitro. Although innovative methods assisted by nanoparticles

are being used in vitro and can be adapted for in vivo applications, few studies have been conducted in livestock and aquatic species. For instance, Tonelli et al. [106] first transferred carboxy-functionalized multiwall carbon nanotube (fMWCNT)-complexed DNA into spermatogonial stem cells (SSCs) with higher efficiency and lower cell mortality than electroporation in Nile tilapia. More recently, polyethylenimine (PEI)-coated nanoparticles with carboxylated single-wall carbon nanotubes (SNWTs) successfully delivered CRISPR/Cas9 components into embryos of Pacific white shrimp with editing efficiency of up to 36%, a four-fold increase over lipid-mediated transfection [107]. These innovative, emerging delivery strategies could revolutionize future application of genome editing in aquaculture.

As early as 1996, it was shown that a zinc finger protein domain, coupled to the *FokI* endonuclease domain, acts as a site-specific nuclease that cuts DNA at strictly defined sites in vitro [108]. The first mutagenesis using a pair of ZFNs was performed in *Drosophila* [109], followed by the generation of gene-edited zebrafish [110]. In subsequent years, two economically important fishes, channel catfish and yellow catfish (*Pelteobagrus fulvidraco*), were edited using ZFN technique [111, 112] (Figure 3). However, ZFN-based technology has several drawbacks, including complexity, high cost of protein domain construction, and high frequency of off-target events (unintended gene mutations) [99]. In this context, TALEN-mediated genome editing with high efficiency was developed in 2010 [100, 113], and subsequently has been applied to several aquatic species, including yellow catfish [114], common carp [115], sterlet (*Acipenser ruthenus*) [116], channel catfish [117], Pacific bluefin tuna (*Thunnus orientalis*) [118], kawakawa (*Euthynnus affinis*) [119], and chub mackerel (*Scomber japonicus*) [120] (Table S3).

CRISPR/Cas9-based genome editing has been gaining momentum since 2013. At present, CRISPR/Cas9, as a relatively new technique, has been found to be powerful in modifying the DNA sequence with small indels, enabling base-specific mutagenesis and also for gene replacement [121, 122]. Although CRISPR cassettes were discovered in *Escherichia coli* in 1987 by Ishino et al. [123], the immune mechanism of CRISPR together with the *Cas* gene against viruses was not revealed until 20 years later [102]. Subsequently, it was shown that the CRISPR/Cas immune system can cleave invading DNA (e.g., plasmid and bacteriophage double-stranded DNA (dsDNA)) at specific loci [124]. Two years later, the group led by Doudna and Charpentier re-engineered the Cas9 endonuclease into a more manageable two-component system by fusing the two RNA molecules into a “single-guide RNA” (sgRNA), which, in combination with Cas9, was able to locate and cut target DNA sequences [121]. By manipulating the sgRNA, the artificial Cas9 system could be programmed to target any DNA sequence for editing. This contribution spurred efforts to edit genomes using the modified CRISPR/Cas9 system, and was ultimately recognized by award of the 2020 Nobel Prize in Chemistry [125] (Figure 3). CRISPR/Cas9-mediated genome editing was first applied to edit human cell lines and zebrafish in 2013 [122, 126], proving its applicability in non-bacterial species. Currently, use of CRISPR/Cas9 to mutate innate-/growth-related genes has made a breakthrough in obtaining desired traits and is giving rise to precision breeding techniques in crops [127–129]. With respect to the aquaculture sector, relevant studies are being carried out in aquaculture stocks [130–133].

Compared to ZFNs and TALENs, CRISPR/Cas9 is the most powerful and widely used editor for genome editing/gene replacement in aquatic species for enhancement of production traits. To date, only 7 of 44 aquaculture species were edited using ZFNs or TALENs, and the rest with CRISPR (Table S3), demonstrating the strong cross-species generalizability of CRISPR due to its versatility, cost-effectiveness, and relative ease of design.

Numerous Cas variants and CRISPR systems have since been engineered for genome editing due to their broad versatility [134]. For instance, the nucleases Cas12a and Cas13 have been characterized in the CRISPR-Cpf1 and CRISPR-C2c2 systems, respectively [135, 136], which are distinct from Cas9. Cas12a produces a ‘staggered’ cut in dsDNA, as opposed to the ‘blunt’ cut caused by Cas9 and requires only one CRISPR RNA (crRNA) for successful targeting. Compared to Cas9 and Cas12a, Cas13 is an RNA-guided RNA endonuclease, with the target of cleaving single-stranded RNA rather than dsDNA. CRISPR/Cas12a-mediated genome editing was demonstrated in efficient mutation of the zebrafish genome [137]. Recently, CRISPR/Cas12a was first tested in a farmed fish, Atlantic salmon, targeting a pigmentation gene (*slc45a2*) with efficiency of up to 34% [138]. However, López-Porras et al. [139] reported that CRISPR/Cas12a-based genome editing led to a lower mutation efficiency (11%) than CRISPR/Cas9 (31%) in Atlantic cod (*Gadus morhua*). Base editing (BE) and prime editing (PE) are the latest advances in genome editing—offering even greater precision and versatility. BE enables direct point mutations at the target site in genomic DNA (e.g., changing C to T or A to G) or in cellular RNA without causing DSBs [140]. Compared to BE, PE is more complicated and consists of three parts: a prime-editing guide RNA (pegRNA), a modified Cas9 fused to an engineered reverse transcriptase enzyme (Cas9-H840A/M-MLV), and a sgRNA, which can be more precise and perform a wider range of targeted substitutions and indels [141]. Although BE and PE have been applied in plants to improve crop production [128, 129], few aquatic species have yet been edited using base or prime editing. The first base editing was applied to a non-model fish, Atlantic salmon, by Raudstein et al. [142], who created a premature stop codon at the target locus by converting C to T. These new genome editing-based tools could contribute to the rapid progress in genome manipulation and biotechnology for aquaculture in the future.

3 | Genome Manipulation to Improve Performance in Aquaculture

To date, genome manipulation of aquaculture species has encompassed a wide variety of technologies, including RNAi, gene transfer, and genome editing, which allow scientists to alter traits through the precise silencing or insertion/modification of specific genes at the RNA or DNA level. The application of genome manipulation tools holds multifaceted advantages. Compared to traditional breeding approaches, these technologies can directly affect desired traits, including increased growth, improved disease resistance, altered body composition or body color of aquatic species in a short period of time, fostering the development of high-performing aquaculture stocks. For instance, RNAi, gene transfer, and genome editing have been used since the 1980s to improve growth, enhance disease

resistance, control reproduction, increase n-3 fatty acid content, and improve tolerance to low DO in channel catfish (Figure 4).

3.1 | Growth

Growth is a critical trait for aquaculture production, and genome manipulation tools enable the modulation of growth and muscle development of aquaculture stocks. RNAi, transgenesis, and genome editing have been applied to improve the growth of aquaculture species over the past four decades. GH genes and promoters derived from human or fast-growing species can be transferred into the genome of target fish species. To date, various GH genes for growth enhancement have been transferred into a total of 19 aquaculture animals, including channel catfish [84, 143], Arctic charr (*Salvelinus alpinus*) [144], common carp [145–147], Nile tilapia [148, 149], southern white shrimp (*L. schmitti*) [150], and mud loach (*Misgurnus mizolepis*) [151], among others. A variety of growth-rate increases were observed among different GH-transgenic fishes. Salmonid GH-transgenic channel catfish grew 26% faster than their non-transgenic, full-sibling F₁ progeny [143]. Transgenic common carp expressing the grass carp GH (*gcGH*) gene driven by the common carp β-actin promoter showed a 42%–80% improvement in body weight compared to non-transgenic siblings [147]. Transgenic Arctic charr possessing the Atlantic salmon growth hormone 2 (*ssGH2*) gene driven by the CMV promoter were 14 times heavier than their control siblings [144]. Compared to other fish species, transgenic salmonids consistently exhibited significantly higher growth rates, with up to 10-fold changes, such as 2–6-fold in Atlantic salmon [152], 6-fold in Chinook salmon (*Oncorhynchus tshawytscha*), 3–10-fold in coho salmon, 6-fold in cutthroat trout (*O. clarkii*), and 3.2-fold in rainbow trout (*O. mykiss*) [89]. Despite the fact that these salmonids carry the same GH transgene (Chinook salmon GH (*csGH*)) driven by a promoter from the ocean pout (*Macrozoarces americanus*) anti-freeze polypeptide (opAFP), the growth rates differed between species. These GH transgene-induced growth differences may be due to species/strain, genomic localization of the transgene, epigenetic, or genetic background coupled with epistatic interactions with other loci [4, 37]. However, the increased growth in GH crustaceans tends to be small, such as the 32% growth increase observed in the southern white shrimp when the tilapia GH (*tiGH*) gene was integrated into the genome [150].

In addition to overexpressing the GH gene by transgenesis for cross-species growth improvement, disruption of growth-regulating genes using genome-editing tools can rapidly increase growth rate in aquaculture species. Myostatin (MSTN), also known as growth differentiation factor 8, belongs to the transforming growth factor-β superfamily and acts on muscle cells to inhibit muscle growth, and is produced by myocytes [153]. Animals either lacking MSTN or blocking the activity of MSTN have significantly more muscle mass. Furthermore, individuals mutated in the *mstn* gene are heavier and stronger than normal [154, 155]. The *mstn* gene, which encodes MSTN, was discovered in 1997 by McPherron et al. [156], who produced a knockout strain of mice that lacked the gene and had approximately twice as much muscle mass as wild-type (WT) mice. As one of the most frequently studied genes for growth enhancement, a total of 11 aquaculture species, including yellow

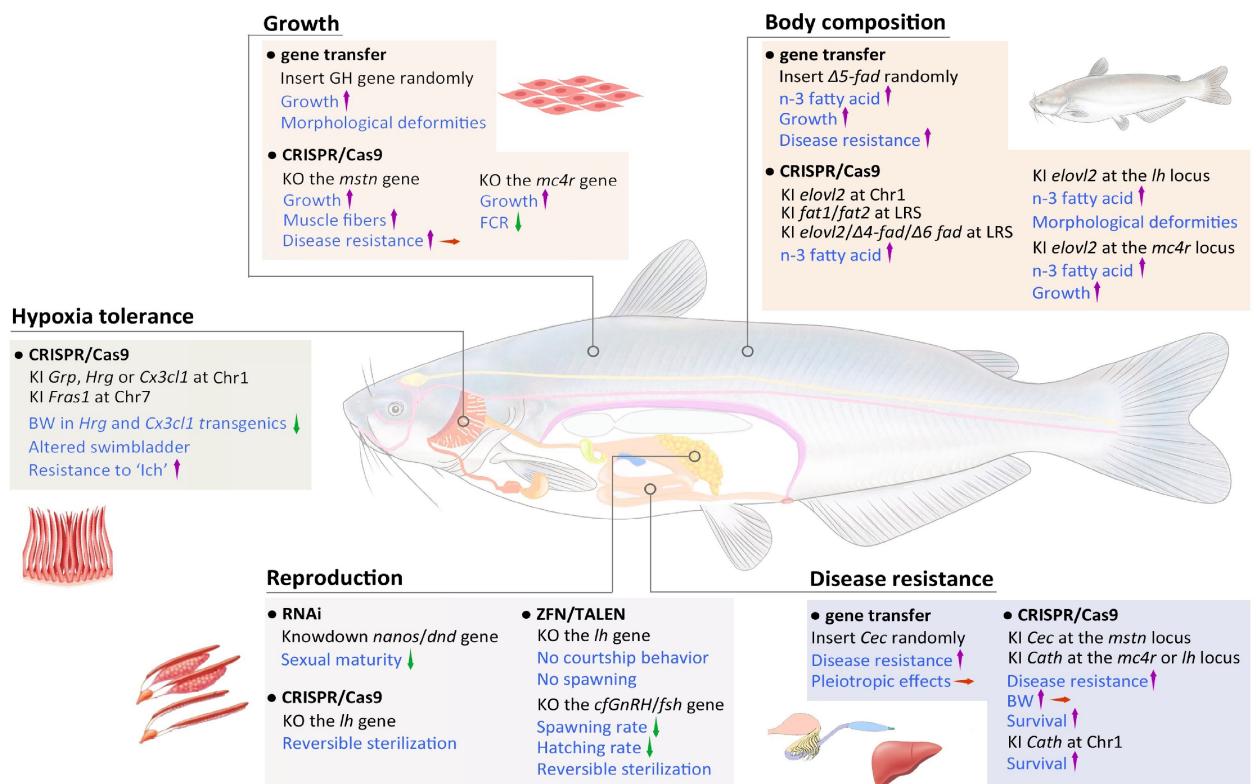


FIGURE 4 | Overview of improved performance by harnessing genome manipulation in channel catfish (*Ictalurus punctatus*). RNAi, RNA interference; ZFN, Zinc-finger nuclease; TALEN, transcription activator-like effector nuclease; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9; KI, knock in; KO, knock out; BW, body weight; FCR, feed conversion ratio; LRS, long repeated DNA target sequence; Chr1/7, chromosome 1/7; Ich, *Ichthyophthirius multifiliis*; GH, growth hormone; *mstn*, myostatin; *mc4r*, melanocortin 4 receptor; $\Delta 4\text{/}\Delta 5\text{/}\Delta 6\text{-fad}$, delta-4/5/6 desaturase; *elovl2*, elongase of very-long-chain fatty acids 2; *fat1*, humanized omega-3 desaturase, encodes a desaturase enzyme that converts n-6 to n-3 fatty acids; *fat2*, encodes the delta12 fatty acid desaturase from *Caenorhabditis elegans*; *lh*, luteinizing hormone; *cGnRH*, catfish gonadotropin-releasing hormone; *fsh*, follicle-stimulating hormone; *nanos*, encodes a small family of evolutionarily conserved RNA-binding proteins that are required for germ cell development and embryonic patterning in diverse organisms; *dnd*, dead end, a germ plasm-specific maternal RNA, *dnd* protein is essential for migration and motility of primordial germ cells; *Grp*, gastrin-releasing peptide-like isoform X2; *Hrg*, histidine-rich glycoprotein; *Cx3cl1*, chromo domain-containing protein cec-1-like isoform X2; *Fras1*, fraser extracellular matrix complex subunit 1; *Cec*, cecropin; *Cath*, cathelicidin. '↑', increased levels; '↓', decreased levels; '→', unchanged levels.

catfish [111, 114, 157], common carp [88, 115], channel catfish [158, 159], olive flounder (*Paralichthys olivaceus*) [160] and red sea bream [161, 162], among others, have shown increased muscle growth through disruption of the *mstn* gene. These studies demonstrated that the deficiency of *mstn* gene contributed to the growth enhancement in fish by reducing *mstn* mRNA levels, increasing the number or size of muscle fibers, improving condition factor and specific growth rate, and up-regulating the expression of myogenesis and lipogenesis-related genes.

RNAi was also adapted to generate *mstn*-suppressed genotypes for growth studies in zebrafish and other non-model fishes. Acosta et al. [163] reported that *mstn*-KD zebrafish exhibited a 39% increase in mean body weight compared to WT individuals at 75 days post-injection. Although the *mstn* gene was knocked down using RNAi in the red abalone (*Haliotis rufescens*), no phenotype or growth data were reported [70]. Until recently, a *mstn*-KD strain of Nile tilapia with increased body weight/length was generated by antisense RNA-based RNAi. Additionally, fish with suppressed *mstn* expression exhibited abnormal lipid metabolism, which contributed to muscle development and body weight [69]. Compared to the disruption of *mstn* by genome

editing, RNAi-induced suppression of the *mstn* at the RNA level led to a more direct effect on gene function, as reflected by a higher improvement in body weight. The average body weight of *mstn*-mutant teleosts was usually 10%–15% to 30%–35% higher than WT groups, which is comparable to results of mammals (15%–30%) [164, 165], although the maximum body weight of *mstn*-null yellow catfish can reach 1.37-fold higher than WT siblings at 210 days post-fertilization [157]. For instance, the mean body weight of *mstn*-mutated channel catfish was increased by 27%–38%, accompanied by a 33.7% or 2% increase in the number or diameter of muscle fibers, respectively, as compared to WT controls [158, 159]. The *mstn*-edited red sea bream exhibited an increase of 10%–16% in muscle mass [161, 162]. Similarly, an 8% increase in body weight and a 27% increase in muscle fiber size were observed in *mstn*-disrupted blunt snout bream (*Megalobrama amblycephala*) [166]. Recently, Wu et al. [167] demonstrated that *mstn*-edited Nile tilapia had a 23% increase in growth rate and an 8% increase in muscle fiber number compared to their WT siblings at 5 months post-hatch. By comparison, *mstn*-KD tilapia produced through RNAi showed 41% and 100% increases in growth rate and muscle fiber number, respectively, at 180-days of age compared to WT fish [69]. In addition

to the genetic background contributing to these differences, one possible explanation is that some small indels of DNA sequences may not result in complete loss-of-function for MSTN protein or may not inhibit *mstn* expression. Wang et al. [168] predicted MSTN structure using the AlphaFold software, and the results indicated that mutations in *mstn* exon 1 had a low probability of causing a structural alteration of the MSTN protein. Another possibility is that the existence of mosaic or heterozygous individuals attenuates the weight gain of gene-edited individuals. In addition, steroidogenic factor 1 (SF₁) plays an important role in gonad development. Cao et al. [68] suggested that downregulation of the *sf1* gene via RNAi not only decreased the gonadosomatic index (by 55%–86%), but also increased body weight (by 34%–45%). Hence, even though insertion of GH transgene by gene transfer and disruption of *mstn* gene by genome editing have more frequently been used for growth enhancement in aquaculture, silencing of growth-related genes using the RNAi technique may also be promising.

Another growth regulator is the melanocortin 4 receptor (MC₄R), encoded by the *mc4r* gene [169]. MC₄R has been found to be involved in feeding behavior, metabolic regulation, and sexual behavior [170, 171]. In fishes, mutations of *mc4r* resulted in increased food intake and decreased metabolism/energy expenditure [172, 173]. For example, adult *mc4r*-KO zebrafish showed increased food consumption/growth and a higher percentage of body fat than WT individuals [174]. Regarding economically important fishes, the *mc4r* of channel catfish was mutated by CRISPR/Cas9-mediated genome editing. Channel catfish with *mc4r* mutations showed 20%–38% weight improvement over the full-sib controls [175], and mutant F₁ progeny were 40% larger than non-edited WT individuals [176], indicating that offspring can inherit target mutations and exhibit improved growth. Coogan et al. [176] observed that homozygous mutants of the *mc4r* gene in F₁ channel catfish grew 30% faster than heterozygotes and controls, indicating that in regard to growth, this phenotype was recessive. What is more, an improved feed conversion ratio (FCR) can be achieved by KO of the *mc4r* gene, which contributes to increased growth. The FCR was improved from 1.57 in the WT controls to 1.18 in the *mc4r*-edited channel catfish [175]. Previous studies revealed that P₁ *mstn*-null channel catfish were 88% heavier than non-edited fish at market size, while the *mc4r*-mutant fish had up to 38% improvement in growth [159, 175]. Our latest data showed that *mstn*-KO channel catfish had increases in body weight, up to 90% and 45% at 3- and 24-month post-fertilization, respectively, whereas this improvement was 76% and 31% in *mc4r*-KO fish, respectively [177]. These findings indicate that *mstn* mutants have a greater increase in growth than did *mc4r*-mutated fish, demonstrating that *mc4r* is a useful candidate gene for improving growth and feed conversion traits in aquaculture due to its large effects on body weight and energy homeostasis. To better compare the effects of *mstn* and *mc4r* mutations on growth rate enhancement, further studies are required to elucidate the mechanism of these two genes for growth regulation in particular fish species and more generally.

Leptin, a satiety factor, plays a critical role in controlling appetite and energy metabolism, which is regulated by the leptin receptor (LEPR) in mammals [178, 179]. *Lepr*-deficient mice have a hyperphagic and obese phenotype with diabetes [180],

supporting the interpretation of LEPR mediating the effects of leptin on energy homeostasis. Since leptin was first identified in pufferfish (*Takifugu rupribes*) [181], subsequent studies have isolated or characterized various leptins in fishes such as common carp [182], zebrafish [183], medaka (*Oryzias latipes*) [184], Atlantic salmon [185], and channel catfish [186]. The role of leptin remained unclear in teleosts until recently, when Yan et al. [187] reported that leptin inhibited feeding behavior and reduced food intake following injection of recombinant leptin-AI/AII in goldfish. In addition, *lepr*-KO medaka exhibited hyperphagia in the post-juvenile and adult stages, leading to a higher growth rate than that of WT controls [188]. In another study, transcriptional analysis showed that the *lepr* gene was involved in the pathways of appetite, sensing, and digestion, and that *lepr*-deficient zebrafish had a lower metabolic rate than WT fish at the larval stage [189]. With respect to farmed fishes, the *lepr* gene was successfully disrupted in rainbow trout using the CRISPR/Cas9 tool, and *lepr*-edited fish showed a hyperphagic genotype, resulting in a faster growth rate (17%–20% increase) than WT individuals [190], which is consistent with the findings in zebrafish [188]. Compared to GH transgenesis, the mutation of growth-related genes is more promising for enhancement of aquaculture products without the introduction of foreign DNA, which matters in gaining regulatory approval. In the future, more gene-edited fish are expected to be on the table, as the first *lepr*-KO tiger puffer (1.9 times heavier than WT) and olive flounder, and *mstn*-edited red sea bream (1.2 times larger than WT) are being sold as food by a startup in Japan—the Regional Fish Institute (<https://regional.fish/en/genome/>). In this vein, the *mc4r* and *lepr* genes are alternative candidates for manipulation for growth improvement in cultured fishes.

Some native fish species in China are popular aquaculture candidates, but their tiny intermuscular bones (IMBs) make them difficult for large-scale processing and consumption. To overcome this obstacle, bony freshwater fishes, including blunt snout bream, gibel carp (*Carassius gibelio*), crucian carp (*C. auratus*) and topmouth culter (*Culter alburnus*), are being manipulated to generate IMB-free strains through genome editing to improve carcass quality. Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily, and one of their major functions is to induce cartilage and bone formation [191]. In zebrafish, the *bmp6* gene was identified as playing a key role in the development of IMBs. Mutation of *bmp6* triggered TNF-A signaling via the NF-Kβ pathway based on RNA-Seq data, which disrupted the formation of IMBs by inhibiting osteoblast development and promoting osteoclast formation [192]. One year later, *bmp6* was characterized and mutated in two farmed fishes, crucian carp and topmouth culter. The *bmp6*-mutant crucian carp grew faster than the WT genotype and showed a complete absence of IMBs in their F₃ homozygous progeny [193]. Subsequently, IMB-reduced topmouth culter was generated by disrupting the *bmp6* locus using CRISPR/Cas9-assisted genome editing [194]. Another IMB-regulated factor known as runt-related transcription factor 2b, encoded by the *runx2b* gene, plays a vital role in osteoblast differentiation in mammals and zebrafish [195, 196]. Taking advantage of the CRISPR/Cas9 tool, IMB-free blunt snout bream and gibel carp were produced without affecting muscle and skeletal development by knocking out the *runx2b* gene [197, 198]. Therefore, IMB-free fishes provide bold innovation and direction for fish

breeding using genome-editing tools, which could make aquaculture more competitive and yield more commercial opportunities by enhancing consumer satisfaction. However, while genetic modification targeting these genes may offer potential benefits, such as controlling unwanted bone formation, it is crucial to carefully evaluate and mitigate potential negative effects on the overall health and growth of the aquaculture species.

3.2 | Disease Resistance

Disease resistance is another key focus of genome manipulation efforts in aquaculture, as pathogens and parasites pose significant threats to aquaculture, leading to economic losses and environmental concerns. Traditional approaches such as chemical treatments, antibiotic use, and vaccines are applied in aquaculture for disease control [13, 132]. However, these methods are limited by environmental concerns or are too labor-intensive for large-scale applications. In contrast, the administration of antibiotics might be reduced with AMPs for disease prevention and control, which could be effective for reducing the use of antibiotics [199]. Alternatively, overexpression of AMP genes by transgenesis or gene replacement using genome-manipulation tools holds promise for the generation of disease-resistant strains with multi-generational heritability [133]. The first fish developed with elevated resistance to bacteria was produced by Dunham et al. [76], who integrated the cecropin (*Cec*) gene from the moth *Hyalophora cecropia* into the genome of channel catfish by electroporation-based gene transfer. The progeny of this transgenic line showed heightened resistance to *Ichthyophthirius multifiliis* (Ich) and *Edwardsiella ictaluri* in their F₃ and F₄ generations, respectively [200, 201]. Chiou et al. [202] reported that *Cec*-transgenic rainbow trout exhibited resistance to infection by *Aeromonas salmonicida* and infectious hematopoietic necrosis virus (IHNV). Grass carp possessing the human lactoferrin (*hLF*) transgene also exhibited higher resistance against GCRV and *A. hydrophila* than their WT counterparts [203, 204]. More recently, the cathelicidin (*Cath*) gene from the Chinese alligator (*Alligator sinensis*) was inserted into the luteinizing hormone (*lh*) locus of channel catfish and blue catfish, and *Cath* transgenics survived *Flavobacterium coviae* or *E. ictaluri* infection at higher rates than their WT controls [79, 81]. To date, a total of nine fish species have been transformed with various AMP transgenes for disease resistance enhancement, showing resistance to 12 infectious diseases. A meta-analysis-based study that the pooled data of these studies indicated that AMP-transgenic fish exhibited enhanced resistance by inducing co-expression between exogenous AMPs and innate immune-related genes [133]. Logistic regression demonstrated that these AMP transgenes regulated the expression of innate AMP genes, such as thrombin-derived C-terminal peptide (*TCP*) and natural killer lysin (*NK-lysin*).

The loss-of-function of disease-susceptibility genes using genome editing is an effective strategy for improving resistance to some diseases, and has been widely applied in crop breeding [128]. However, except for a few related studies conducted in vitro using fish cell lines, few aquaculture species have been produced by knocking out disease-susceptibility genes. For example, KO of the junctional adhesion molecule-A (*JAM-A*) gene increased resistance against GCRV by suppressing the cytopathic effect in grass carp kidney cells [205]. In addition, *STAT2*-null embryo

cells from Chinook salmon have been shown to have increased resistance to various viruses [206]. Subsequently, deletion of the *EcBCO2* or *EcNinaB-X1* genes significantly improved resistance to *Vibrio parahaemolyticus* and *A. hydrophila* in oriental prawn (*Exopalaemon carinicauda*) [207, 208]. Recently, genetic resistance to infectious pancreatic necrosis virus (IPNV) in Atlantic salmon was shown to be determined by a single QTL, NEDD-8 activating enzyme 1 (*nael*), based on whole-genome sequencing. KO of *nael* can reduce the incidence of IPNV by inhibiting virus replication in salmon head kidney cells [209]. Therefore, elevating disease resistance by KO of disease-susceptibility genes maybe promising as more disease-related alleles/markers (e.g., QTLs and SNPs) are revealed by RNA-Seq in fish species.

With respect to in-vivo experiments, a few representative genes, such as rhamnose-binding lectin (*RBL*) and GRB2-associated binding protein 3 (*gab3*), have undergone induced mutations that alter the immunity of the fish. RBL is an important component of fish's innate immunity as an antibacterial molecule, and potential negative regulation of RBL against pathogenic invasion has been observed in some fishes [210, 211]. Beck et al. [212] confirmed that columnaris susceptibility was negatively associated with *RBL* expression levels. An *RBL*-null channel catfish line was established [86], and their F₂ progeny had higher survival than WT fish after infection with *F. columnare*. Likewise, GAB proteins have been shown to facilitate replication of human and fish viruses. An in-vivo study demonstrated that KO of *gab3* in zebrafish improved survival following infection with the nervous necrosis virus [213]. Moreover, tripartite motif (TRIM) proteins encoding ubiquitin ligases help regulate the innate immune system. Teleost fishes have multiple TRIM genes, and a previous study identified a TRIM gene, *ftrca1*, from crucian carp. FTRCA1 downregulates the interferon (IFN) response of fish during viral infection by regulating the expression of specific genes, demonstrating that FTRCA1 is a negative regulator of IFN immunity in fish [214]. Furthermore, loss-of-function of *ftr42*, a homolog of *ftrca1*, can improve resistance against spring viraemia of carp virus by enhancing IFN defense in zebrafish [215]. Nonetheless, knowledge of these in-vitro- or in-vivo- verified genes has not been applied to genomic manipulation of farmed fishes or crustaceans. A possible explanation is that these genes are pathogen-specific and have a low, broad-spectrum effect. Of note, several immune-related genes, such as chitinase (*EcChi4*) in oriental prawn [216] and *viperin* in gibel carp [217], were edited using CRISPR/Cas9 to determine their role in immune processes. These *EcChi4*- or *viperin*-deleted individuals showed higher mortality than their full siblings after bacterial or viral infection, demonstrating that these genes are involved in innate immunity.

These transgenesis- and genome-editing-based strategies have only rarely been applied to crustaceans and shellfishes. Alternatively, disease resistance against a wide variety of pathogens, including viruses, bacteria, and parasites, has been improved in crustaceans using RNAi-based genome manipulation tools rather than gene transfer or genome editing. To date, the control of crustacean viral diseases by RNAi based on virus-host interactions has been a field of intense research, since the initial use of RNAi approach to control viral diseases in shrimp was introduced in 2004 [60, 218]. There are two main strategies to control viral diseases in shrimp using RNAi-involved treatment:

First, the RNAi molecules (e.g., dsDNA, siRNA, shRNA, and antisense RNA) targeting a specific viral gene are synthesized and injected into shrimp, followed by WT (virulent) virus infection to test for resistance to the virus. Second, the WT virus is RNAi-modified into a low-virulence strain by infecting it with specific RNAi molecules, and then it is injected into shrimp to observe the survival. In both cases, a reduction in mortality will be observed compared to untreated shrimp, as the expression of related genes encoding the viral protein is inhibited (Figure 5A).

At present, Pacific white shrimp and giant tiger prawn are the main species for disease resistance enhancement using RNAi, followed by kuruma shrimp (*Marsupenaeus japonicus*) and oriental river prawn (*Macrobrachium nipponense*). White-spot syndrome virus (WSSV) is the causative agent of white-spot disease, which is one of the most lethal infections in shrimp farming. RNAi tools have been evaluated in shrimp to provide protection against WSSV due to the lack of effective treatment to prevent this virus. Knockdown of WSSV genes, such as structural protein genes (*vp15*, *vp24*, and *vp28*) involved in the viral infection cascades of shrimp, is the major strategy to reduce viral replication in vivo. *vp*-KD shrimp showed significantly higher survival than WT individuals during WSSV challenge. For example, *vp24*- and *vp28*-KD Pacific white shrimp had high survival rates after WSSV infection, and significant reductions in viral titers were observed in the RNAi-treated groups [219, 220]. Silencing

of the *vp15* or *vp28* gene by injection of RNAi molecules, leading to reduced viral load and low mortality, has been reported in kuruma shrimp [221] and giant tiger prawn [53, 61]. In contrast, in some cases, silencing specific innate immunity genes of shrimp is another option to improve resistance to WSSV. Caspase-3 (Cap3), a key protein in the induction of apoptosis, has been shown in previous studies to be associated with apoptosis in WSSV-infected shrimp after viral challenge. *Cap3*-silenced shrimp exhibited high survival (1.9-fold increase) and delayed mean time to death (25% increase) following WSSV infection [222]. In addition to WSSV, infection by other viruses, bacteria, and parasites can be controlled by knocking down a variety of genes in different crustaceans (Table S1).

Two fish species [orange-spotted grouper (*Epinephelus coioides*) and rainbow trout] were experimented upon to enhance disease resistance using RNAi-based tools. Antiviral activity was observed in RNAi-treated rainbow trout, as mortality was reduced 50% following the viral challenge when siRNA was designed to target the viral envelope G gene of viral hemorrhagic septicemia virus (VHSV) [62]. In addition, siRNA-induced RNAi can inhibit whirling disease by interrupting the life cycle of the parasite (*Myxobolus cerebralis*) in rainbow trout [223]. Similarly, the *impB* gene was identified in association with the virulence of *Pseudomonas plecoglossicida* bacteria, and *impB*-silent *P. plecoglossicida* showed no virulence to orange-spotted grouper,

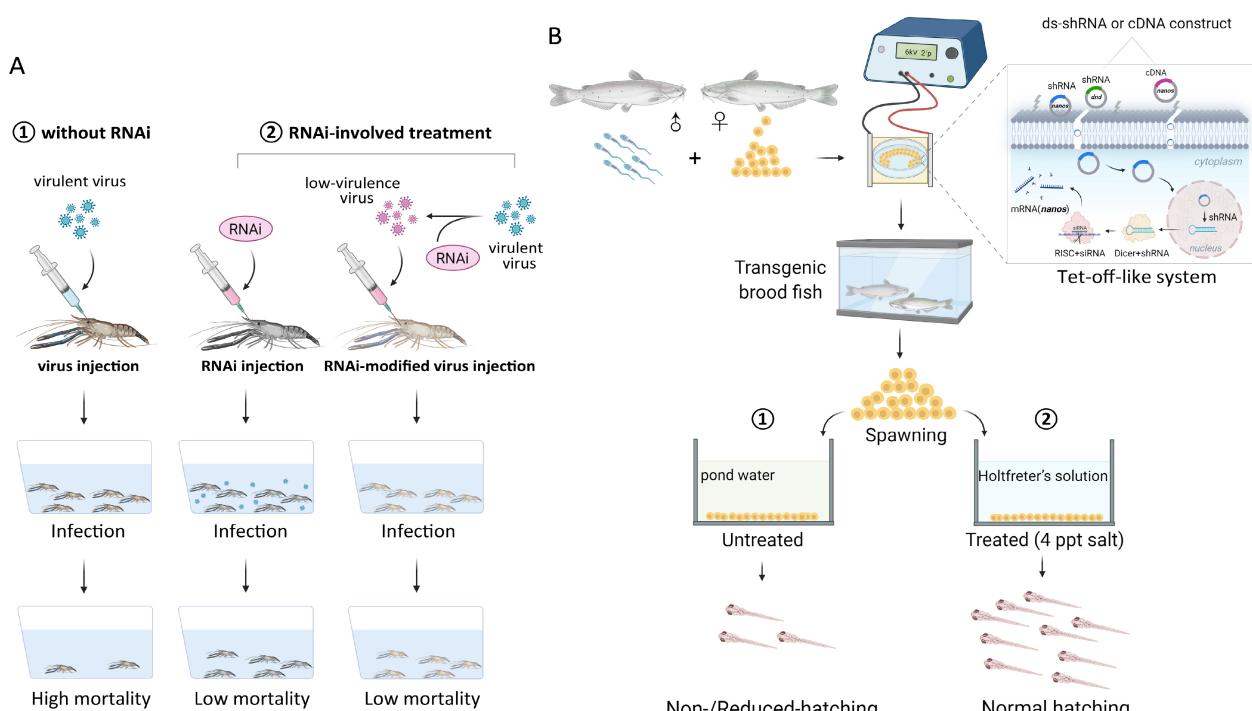


FIGURE 5 | Representative applications of the RNAi tool in crustaceans and fishes. (A) Approaches to improve resistance against viruses by silencing viral structural protein genes using RNAi in crustaceans. Two main strategies are used: RNAi molecules are injected into crustaceans prior to virus infection, then crustaceans are challenged with virulent viruses (WT); alternatively, RNAi molecules are transfected into viruses to produce low-virulence strains (RNAi modified), then crustaceans are challenged with RNAi modified viruses. (B) Generation of transgenic sterilization lines by knocking down primordial germ cell (PGC) marker genes using RNAi in fishes. Salt-sensitive Tet-off-like systems containing ds-shRNA or cDNA are constructed by targeting the 5' end or 3' end of *nanos*, full-length cDNA of *nanos* or *dead end* (*dnd*). The transgenic broodstock can be generated by electroporating WT embryos with designed plasmid constructs, and each construct is divided into an untreated group and a treated group with sodium chloride as the repressor compound. The embryos from P₁ transgenic parents show normal hatching rates with the salt treatment, while no hatching or significantly reduced hatching is observed without salt treatment. WT, wild type; RNAi, RNA interference; ds-shRNA, double-stranded short hairpin RNA; siRNA, small interfering RNA; cDNA, complementary DNA.

as reflected in 100% survival after infection by the *impB*-RNAi strain bacteria [224]. These studies demonstrated that KD of immune-specific genes using RNAi has a broad-spectrum effect against various pathogens, including viruses, bacteria, and parasites, and is applicable to both fishes and crustaceans. However, it is important to note that these applications are primarily for studying gene functions and are not suited for aquaculture breeding or large-scale production.

3.3 | Reproduction

Genetically modified (GM) fishes have great potential to increase production, disease resistance, and other desirable traits. However, the potential for introgression of GM fish into natural populations and ecological implications of escape of these fish into ecosystems have raised genetic and ecological concerns [225]. Effective fish sterilization could eliminate or significantly reduce the environmental threats posed by GM/transgenic fishes [4, 226]. In the context of reproductive confinement, genome editing could block hormones or genes involved in reproduction. The hypothalamic–pituitary–gonadal (HPG) axis is a major signaling pathway that controls gonadal differentiation in fish. Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus by GnRH-expressing neurons in the brain. The anterior part of the pituitary gland produces luteinizing hormone (LH) and follicle-stimulating hormone (FSH) following stimulation by GnRH, and the gonads produce estrogen and testosterone [227, 228]. Therefore, GnRH, FSH and LH are vital and responsible for gonadal maturation in teleosts, which can be manipulated to control reproduction.

FSH and LH were first mutated in zebrafish using TALENs, and *fsh*-deficient zebrafish showed delayed development of ovary and testis. In contrast, *lh*-deficient females were sterile [229, 230]. Subsequently, channel catfish carrying *lh* mutations were generated using ZFN-based genome editing by electroporation. *lh*-null mature fish did not display courtship, and spawning was not observed in natural environments [112, 117]. In parallel, editing of *GnRH* and *fsh* in channel catfish was accomplished using TALENs to create reproduction-controllable strains. Both *GnRH*- and *fsh*-edited individuals had low fertility, and application of luteinizing hormone-releasing hormone analog (LHRHa) or human chorionic gonadotropin (HCG) hormone therapy restored spawning (up to 60%) and hatching (up to 80%) in these mutants [117]. Additionally, the insertion of an AMP gene into the *lh* locus of channel catfish not only improved disease resistance but also sterilized fish, and this reversal fertility can be restored by the induction by an LHRHa/HCG mixture [81]. More recently, Andersson et al. [231] suggested that disruption of the FSH receptor (*fshr*) gene leaves males immature with small testes, resulting in male infertility in Atlantic salmon.

Fish sterility can also be achieved experimentally by inactivating mRNAs essential for primordial germ cell (PGC) formation, such as *dnd*, as shown in zebrafish and medaka [232, 233]. Since the generation of the first *dnd*-KO Atlantic salmon [234, 235], *dnd* mutants have been generated using CRISPR/Cas9-based genome editing in a variety of fish species, including rice field eel (*Monopterus albus*) [236], sterlet [237], and striped catfish (*Pangasianodon hypophthalmus*) [238]. These *dnd*-edited fish

exhibited a reduced number of PGCs and a low gonadosomatic index, indicating an obstruction of gonadal development. Another key protein-encoding gene, P-element induced wimpy testis (*piwi*), is essential for germline survival. Disruption of *piwil2* and *piwil1* resulted in different resultant genotypes in Nile tilapia and Atlantic salmon, respectively. *piwil2*-KO tilapia had no/fewer PGCs compared to WT fish in the P₁ generation [239], whereas *piwil1*-mutant P₁ Atlantic salmon still had PGCs and produced F₁ offspring with lacked PGCs [240]. A possible hypothesis for this inconsistent result is that mosaic or heterozygous individuals carrying *piwil2* mutations were presented in P₁ Atlantic salmon. Another possible explanation is that Atlantic salmon are tetraploid, and only the knockout of all copies of the target gene can cause the total loss of function. Intriguingly, several studies suggest that sex reversal can be achieved by knocking out a single gene, such as PDZ domain-containing (*pfpdz1*), forkhead box protein L2 (*foxl2*), and cytochrome P450 17A1 (*cyp17a1*) genes in different fishes. Dan et al. [241] revealed that inactivation of *pfpdz1* by genome editing triggered ovarian differentiation in yellow catfish, while overexpression of *pfpdz1* by transgenesis induced XX ovaries to differentiate into testicular-like tissues. Multiple *foxl2* homeologs were edited using CRISPR/Cas9 in gibel carp, and approximately 30% of mutant females completely reverted to males [242]. Recently, the depletion of *cyp17a1* was shown to produce *cyp17a1*^{-/-} XX neomales, which could be used to generate all-female populations (*cyp17a1*^{+/+} XX) through neomale-WT female mating [243]. In the ridgetail white prawn (*Exopalaemon carinicauda*), sex-reversed neo-female individuals with female phenotypic characteristics can be obtained by knocking out the insulin-like androgenic gland (*IAG*) hormone gene using CRISPR/Cas9 [244]. These genes are promising candidates for generating monosex teleost/crustacean populations in sexually dimorphic species.

Encouragingly, as an important model fish for reproduction studies, an increasing number of sex-determining genes have been identified and functionalized in Nile tilapia using CRISPR/Cas9 genome editing. In males, knockout of specific reproduction-related genes resulted in decreased spermatogenic cells or reduced sperm quality/motility and even sterilization. For instance, CRISPR/Cas9-mediated mutation of eukaryotic elongation factor 1 alpha (*eEF1A*) resulted in infertility in P₁ males due to abnormal spermiogenesis [245]. Male tilapia carrying the estrogen receptor (*esr*) mutation had smaller testis, fewer spermatogonia, and more abnormal sperm than WT controls [246]. Moreover, the insulin-like growth factor (*igf3*) gene has been shown to regulate spermatogonial proliferation and differentiation in fishes. *Igf3*-mutant XY tilapia were subfertile, with drastically reduced semen volume and sperm count [247]. Similarly, disruption of the thrombospondin1 (*tsp1*) and *esr2b* genes resulted in delays in ovarian development in females [246, 248]. In addition, sex reversal in tilapia can be achieved by editing the steroidogenic factor-1 (*Sf-1*) gene, which regulates steroidogenesis and reproduction in mammals. *Sf-1*-deficient male Nile tilapia displayed gonadal dysgenesis and feminization, and both *sf-1*^{+/−} F₁ XX and XY mutants developed as fertile males. *Sf-1* deficiency also resulted in female-to-male sex reversal in 8.1% of F₀ and 92.1% of *sf-1*^{+/−} F₁ in XX individuals [249]. Nonetheless, the evaluation of key sex-determination genes from these studies was inconclusive. These potentially

functional genes would need to be further validated and selected for large-scale application of reproductive control in tilapia and other aquaculture species.

An alternative to the knockout of reproduction-related genes for genetic control of reproduction is RNAi-mediated gene suppression. RNAi can be used to downregulate or silence genes essential for reproductive processes in aquatic species, especially crustaceans. The hormone IAG plays an important role in sexual differentiation and maintenance of masculinity in crustaceans. In this vein, a sexual shift was observed by silencing the *IAG* gene in numerous studies, with varying outcomes across different species. For instance, *IAG* silencing by dsRNA injection in the red-claw crayfish (*Cherax quadricarinatus*) induced feminization of male traits or secondary sexual characters, sperm reduction, and testicular degeneration [250], consistent with results of subsequent studies in the giant river prawn. *IAG*-silenced giant river prawn displayed delayed appearance of male-specific secondary traits and induced neo-females with ovarian development [65, 251]. In addition, *IAG* silencing by RNAi has been performed in other species, including Chinese mitten crab (*Eriocheir sinensis*) [67], peppermint shrimp (*Lysmata vittata*) [252], and red swamp crayfish (*Procambarus clarkii*) [253, 254]. Sharabi et al. [66] found that RNAi-mediated suppression of the insulin-like receptor (*IR*) increased *IAG* production and accelerated the appearance of male secondary sexual characters by blocking the *IAG* synthesis pathway. In addition, RNAi has found a niche application in aquaculture, particularly in the sex reversal of species such as shrimps and prawns. For example, in the freshwater prawn *Macrobrachium rosenbergii*, RNAi has been used to reverse sex, producing all-female monosex populations. This approach is beneficial because all-female populations often show better growth rates and are more desirable in commercial production due to their uniformity and higher market value. A successful large-scale field study demonstrated the effectiveness of this method [255], where the RNAi-induced sex-reversal technique was employed to produce all-female monosex cultures, significantly improving production efficiency in prawn farming.

In fishes, PGC marker genes, including *nanos* and *dnd*, have been silenced using RNAi tools in channel catfish and common carp to produce sterile strains. Previous studies have demonstrated that KO of *nanos* and *dnd* using genome editing can generate PGC-less strain of Nile tilapia and Atlantic salmon, respectively [234, 256]. Theoretically, similar outcomes could be obtained in other fish species by suppressing the expression of these two genes via RNAi. Knockdown constructs (driven by a copper transport protein gene) based on 3' *nanos* short hairpin RNAi appeared to result in the best suppression and subsequent restoration of normal sexual maturity in channel catfish [51, 257], and reduced gonad development (93% smaller than WT) in PGC-KD fish [258]. To mitigate the potential risks of copper, a salt-sensitive Tet-off-like system (driven by a salt-sensitive promoter) was constructed to knock down *nanos* and *dnd* in channel catfish (Figure 5B). In this design, salt-treated embryos repressed expression of the KD construct and allowed PGC to proliferate for gonadal development of broodstock. The spawning rates of full-sibling P_1 channel catfish with and without exposure to the construct as treated and untreated embryos were 93% and 59%, respectively [52]. In common carp, four constructs

containing RNAi molecules were electroporated into embryos to KD or eliminate expression of two PGC genes, *nanos* and *dnd*. The targeted mRNAs of *nanos* and *dnd* were knocked down to minimal levels or degraded, resulting in reduced rates of sexual maturation in males [64]. These plasmid-assisted RNAi strategies are promising and applicable for reversible sterilization in fishes without induction of foreign genes.

3.4 | Body Composition

Biotechnological alteration of the nutritional profile of fish could be beneficial to consumers, and it is now possible to directly alter body composition through transgenesis or gene replacement. Omega-3 polyunsaturated fatty acids (n-3 PUFAs), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to have significant benefits for human health [259]. However, not all fish species have high levels of n-3 PUFAs. Therefore, one of the major goals of genome manipulation is to enhance the levels of EPA and DHA in farmed fishes that are low in these fatty acids. Zebrafish transformed with $\Delta 5$ - or $\Delta 6$ -desaturase-like genes from masu salmon (*O. masou*) had increased levels of DHA and EPA in their flesh compared to non-transgenic individuals [260, 261]. A β -actin–salmon $\Delta 5$ -desaturase-like gene was inserted into the genome of common carp, and the P_1 transgenic fish showed 1.1-fold higher EPA/DHA than the non-transgenic fish [262].

As such, some improvements have been accomplished in recent years by inserting genes involved in EPA/DHA biosynthesis into channel catfish. Huang et al. [263] reported that the higher ratio of n-3 fatty acids observed in the β -actin–salmon $\Delta 5$ -desaturase transgenic channel catfish in the F_1 generation (1.25-fold and 1.34-fold in females and males, respectively), enrichment similar (1.11-fold) to results of another study suggesting F_1 $\Delta 5$ -desaturase-transgenic channel catfish [264]. In addition to desaturase genes, the elongase-like genes, such as the elongase gene for producing very-long-chain fatty acids (*elovl2*), plays a vital role in the DHA biosynthesis pathway [265]. Overexpression of the elongase-like gene (*melo*) from masu salmon in transgenic zebrafish improved the DHA content by 1.33-fold compared to non-transgenic fish [266]. In addition, transgenic nibe croaker (*Nibea mitsukurii*) carrying the *elovl2* gene isolated from masu salmon, had 2.28-fold higher docosapentaenoic acid (DPA) content than their non-transgenic counterparts [267], which can be converted to DHA. Building on these previous studies, Xing et al. [78, 268] produced *elovl2*-transgenic channel catfish with DHA content in muscle up to 1.62-fold higher than WT full siblings. The transgenic channel catfish not only showed an increase of 121.6% and 32.8% in EPA and DHA, respectively, but also had a 41.8% increase in body weight compared to non-transgenic fish when the *elovl2* transgene was inserted into the *mc4r* locus [269]. Another gene that encodes a desaturase enzyme (omega-3 desaturase) is *fat1*, which exists in lower lifeforms, such as plants, microorganisms, and nematodes but is absent in most animals [270, 271]. Previous studies have demonstrated that *fat1*-transgenic zebrafish and common carp displayed a great improvement in n-3 fatty acids and a dramatic increase in the n-3/n-6 ratio [272, 273]. In this vein, multiple copies of the *fat1* transgene were integrated into the genome of channel catfish, and DHA and n-3/n-6 ratio

were significantly increased by 37% and 25.2%, respectively, in *fat1*-transgenic fish compared to WT controls [274]. These studies demonstrated the feasibility of dramatically improving n-3 PUFAs in aquaculture species by overexpressing the EPA/DHA biosynthesis-related genes.

3.5 | Environmental Adaptability

Most efforts to develop transgenic fish have been devoted to growth enhancement and disease resistance, although there also are reports of improvements in cold or hypoxia tolerance. One of the possible explanations for the lesser effort to explore environmental tolerances using gene transfer is that many hybrid fishes in early studies show increased environmental adaptability due to heterosis. For example, the hybrids between Nile tilapia (low salinity tolerance) and red tilapia, *O. mossambicus* (high salinity tolerance), displayed improved tolerance to high salinity [275]. Red tilapia backcrossed to cold-tolerant blue tilapia, *O. aureus*, and the F₁ backcross showed better cold tolerance than either parent [276]. Interspecific channel × blue hybrid catfish exhibited a 50%–100% increase of heterotic tolerance to low oxygen [277, 278]. In addition, MAS proved effective for increasing tolerance to extreme temperatures. In rainbow trout, 25% of progeny exhibited a heightened degree of upper-temperature tolerance following MAS for heat tolerance [279]. While some GH-transgenic fished did show improved tolerance to low temperature or low oxygen, these improvement abilities were mainly due to positive pleotropic effects of the transgenes. For example, GH has a critical role in osmoregulation and ventilation [4, 280], which could be related to the response to conditions of low oxygen. GH-transgenic common carp and channel catfish showed better tolerance to oxygen stress coupled with lower ventilation rates at low DO compared with controls.

Early research also involved the transfer of AFP from the winter flounder (*Pseudopleuronectes americanus*) into Atlantic salmon to produce freeze-resistant salmonids that could be farmed under sub-Arctic conditions [281]. Enhanced tolerance to low temperatures was observed by injecting AFPs into rainbow trout [282], indicating that AFPs were responsible for the ability of fish to survive super-cooled seawater. Subsequently, the linearized AFP-encoding DNA isolated from winter flounder was inserted into the genome of Atlantic salmon, but no low-temperature challenge experiments were conducted to verify cold tolerance [281]. Preliminary results with goldfish showed some promise for increasing survival within the normal cold temperature range. Transgenic goldfish carrying the AFP gene from ocean pout were significantly more tolerant than WT controls when challenged with low temperatures [283].

Improving hypoxia tolerance via gene transfer/replacement has been only minimally attempted in aquaculture species. With the advent of RNA-Seq technology, some genes related to hypoxia tolerance have been revealed in some species, which provides a path to improve hypoxia tolerance in hypoxia-intolerant species. Previous studies have shown that hypoxia tolerance involves the hypoxia-inducible factor (HIF) signaling pathway, and the factor inhibiting HIF (FIH) inhibits the transcriptional activation of hypoxia-inducible genes by blocking the association of HIFs with the transcriptional coactivator CREB-binding protein

[284, 285]. In this regard, Cai et al. [286] proved that KO of *fib* made zebrafish more tolerant to hypoxic conditions than WT siblings by activating transcription of HIF proteins. However, such strategies aimed at knocking out HIF genes to improve hypoxia tolerance have not been applied to any economically important species. Alternatively, key genes for the formation of accessory air-breathing organs (ABO) in two air-breathing catfish species, bighead catfish (*Clarias macrocephalus*) and tra catfish (*Pangasianodon hypophthalmus*), were identified and characterized using RNA-Seq analysis. Based on comparative genomic analysis, the *Grp*, *Cx3c11*, *Hrg* genes in tra catfish and the *Fras1* gene in bighead catfish were identified and verified as the most likely contributors to the formation of swimbladder and air-breathing [287]. Subsequently, these genes driven by their native promoters were transferred into the genome of channel catfish in an attempt to modify their response to hypoxia using the CRISPR/Cas9 system. *Grp* transgenics showed alterations in the swimbladder morphology [288], which expands the knowledge base on the adaptation of aquatic organisms to hypoxia, as well as preliminary insights into the production of ABO transgenic fish capable of surviving in low-oxygen water.

3.6 | Pigmentation

Early research on pigmentation was primarily concerned with using transgenesis to produce fish with desired pigmentation patterns for aesthetic or commercial purposes. In ornamental fishes, transgenesis is often used to introduce genes that produce the GFP, YFP and RFP (green, yellow and red fluorescent proteins, respectively), resulting in fish that glow in various colors under ultraviolet light. For example, the GloFish lines of genetically engineered fishes (<https://www.glofish.com/>), including zebrafish, skirt tetra (*Gymnocorymbus ternetzi*), tiger barb (*Puntius tetrazona*), rainbow shark (*Epalzeorhynchos frenatum*), Siamese fighting fish (*Betta splendens*), and bronze corydoras (*Corydoras aeneus*) with fluorescent colors are on the U.S. market [289]. Now that ornamental fish market is becoming saturated in the U.S. market, new transgenic aquatic species carrying fluorescent proteins rarely appear.

Instead, recent work in pigment gene knockouts has primarily served to demonstrate the feasibility of gene editing or to develop effective genome-editing tools, as changes in body color or albinism are effective indicators of editing success. The most frequently studied genes are solute carrier family 45 member 2 (*slc45a2*) and tyrosinase (*tyr*), as they are evolutionarily conserved mediators of melanin biosynthesis [290, 291], and *slc45a2*- or *tyr*-mutant genetic lines have been generated in several farmed species in many studies, including Atlantic cod (*Gadus morhua*) [139], Atlantic salmon [138, 142, 292], goldfish [293], large scale loach (*Paramisgurnus dabryanus*) [294], large yellow croaker [295], kawakawa (*Euthynnus affinis*) [119], Nile tilapia [296], Oujiang color common carp (*Cyprinus carpio* var. *color*) [297], sterlet [298] and white crucian carp (*Carassius auratus cuvieri*) [299]. Although most *slc45a2*- or *tyr*-mutant fishes have displayed reduced pigment cells or albino-like phenotypes, *tyr*-null large yellow croakers did not show obvious albinism [295], consistent with results of a previous study in zebrafish [300]. The possible reason for this is that neutral/silent mutations are detected in the

targeted DNA sequences, resulting in no alterations in protein structures. Of importance, the albino gene enabling direct visual evaluation is commonly used as an indicator to test the editing efficiency of novel genome-editing tools. For example, *slc45a2* was targeted by the Cas12a protein in Atlantic salmon and Atlantic cod to assess gene-editing efficiency, and showed lower efficiency and fewer chromatophores reduced compared to Cas9-mediated editing [138, 139]. In addition, a high rate of loss-of-function in *slc45a2* coupled with a significant reduction in melanin was observed in Atlantic salmon using base editing [142], indicating the flexibility of using base editing in non-model fishes.

In addition to *slc45a2*- and *tyr*, the melanocortin 1 receptor (*mc1r*) gene plays an important role in melanin modulation in fish species, as it is the major receptor for the agouti-signaling protein (ASIP) [301]. *Mc1r* loss-of-function mutant zebrafish showed significant differences in body color pattern, accompanied by a lower density of melanophores in the dorsal region, lateral region and belly than WT fish [302]. Disruption of *mc1r* in Oujiang color common carp produced shrunken and underdeveloped melanophores in the skin, leading to albinistic skin [303]. Editing of other genes, such as oculocutaneous albinism II (*oca2*) in cavefish (*Astyanax mexicanus*) and yellow river carp (*Cyprinus carpio haematopterus*) [304, 305], tryptophan-2,3-dioxygenase (*tdo*) in longfin inshore squid (*Doryteuthis pealeii*) [306], premelanosome (*pmel*) in Nile tilapia [307], golden (*gol*) in Northeast Chinese lamprey (*Lethenteron morii*) [308] and ASIP in Oujiang color common carp [309] produced varying degrees of albino-like or reduced melanophore phenotypes by blocking/disrupting the melanin synthesis pathway. They are promising candidates for future applications to produce albino-like phenotypes.

3.7 | Behavior

Genome editing in aquaculture also has the potential to significantly influence the behavior of aquatic organisms. A typical example is the *lepr* gene, and *lepr*-KO zebrafish and rainbow trout behaved hyperphagically, indicating that *lepr* controls food intake and digestion, which contributes to fast growth [189, 190]. Intriguingly, L-amino acid intake and feeding behavior are associated with the gene taste receptor family 1 (*t1r*) in mammals. The conserved function of *t1r* or its heterodimers (*t1r1* and *t1r3*) has been validated in fishes, including medaka and grass carp [310, 311]. A recent study showed that *t1r1*_KO zebrafish had a broadened diet, showing that mutant fish not only consumed the usual foods (e.g., *Artemia*, insect larvae), but also accepted aquatic plants (e.g., duckweed) that were rejected by WT zebrafish [312]. This strategy to control fish intake could be useful for the generation of new strains with desired fatty acids in aquaculture species.

In tuna farming, high-speed swimming induced by sudden changes in light intensity leads to wall collisions, resulting in high mortality of hatchery-reared juveniles in sea cages [313]. One possible way to reduce mortality is to reduce swimming speed, which is associated with muscle contraction in skeletal muscle. Recently, a ryanodine receptor 1 (RyR1, encoded by the *ryr1b* gene) mutant zebrafish line displayed weak muscle

contractions and slow swimming in response to external stimuli, implicating that *ryr1b* is a key gene involved in fast swimming [314]. As such, targeted mutagenesis of the *ryr1b* gene using TALEN-mediated genome editing resulted in slow swimming behavior in the larval stage of Pacific bluefin tuna (*Thunnus orientalis*) [118], which would be beneficial for creating genetically modified strains with special traits in the culture of fast-swimming species. Similarly, β -tubulin-KO larval Fujian oyster (*Crassostrea angulate*) and Pacific abalone (*H. discus han-nai*) exhibited decreased motility due to defective cilia [315, 316]. However, these studies focused on using the β -tubulin gene as a marker for successful genome editing rather than for breeding applications in shellfish.

In addition, some fish species exhibit complex social behaviors, including dominance hierarchies or cooperative interactions. Genome editing could be used to manipulate genes involved in social behavior, potentially altering social dynamics within fish populations. For example, reducing aggression or increasing social tolerance could lead to more harmonious group interactions, minimizing stress and improving overall welfare. Androgens such as testosterone, regulated by the androgen receptor (AR), are strongly linked to social status and reproductive behavior in a model fish, African cichlid *Astatotilapia burtoni*. African cichlids carrying the AR mutations exhibited atrophic testes, darkened body coloration, and reduced aggressive displays [317]. Moreover, the neuropeptide arginine vasotocin (AVT) is a key modulator of affiliation and aggression in non-mammals, and previous studies have identified four AVT receptors (V1a1, V1a2, V2a, and V2b) in teleosts [318]. Yokoi et al. [319] demonstrated that KO of the *V1a2* gene significantly reduced the mate-guarding aggression in medaka. In this case, a similar strategy was adopted in scombrid fish to generate an easy-to-rear, manageable strain. Less-aggressive *V1a2*-null chub mackerel (*Scomber japonicus*) were produced using TALENs, and this new line showed reduced cannibalistic behavior (46% reduction in frequency) at the fry stage [120], significantly reducing fry mortality under artificial conditions.

4 | Technical and Regulatory Challenges

The emergence of genome manipulation technologies has sparked significant interest in harnessing the potential of aquatic organisms with enhanced traits and improved performance in aquaculture. While the prospect of genetically engineered aquatic species holds great promise for addressing pressing challenges in food security, disease management, and environmental sustainability, it also presents many complex challenges and considerations that must be carefully navigated.

4.1 | Technical Challenges

Off-target effects are the major concern of genome manipulation in aquaculture applications. Off-target events can be observed in both RNAi and genome-editing experiments. Although few studies have reported off-target effects of RNAi in aquatic species, they can occur when the RNAi machinery silences genes other than the intended target. This can happen if the siRNA or miRNA sequences used for RNAi share partial homology

with unintended mRNA targets [72]. Su et al. [51] observed that off-target events caused by RNAi in channel catfish resulted in lower hatch and fry survival rates compared to controls. Moreover, expression of other PGC marker genes, such as *vasa*, showed 1.5- to 11.3-fold off-target downregulation when RNAi molecules were designed to target the *dnd* and *nanos* genes [52, 257]. Also, overdoses of exogenous siRNAs or miRNAs may saturate the endogenous RNAi machinery, leading to competition for binding sites and reducing the efficiency of specific gene silencing. This may result in incomplete KD of the target gene or interference with other endogenous RNAi processes that are essential for normal function within the organism. In practical applications, immersion or oral administration of exogenous RNAi molecules can affect non-target species through water-borne transmission or consumption of RNAi-treated feed, potentially affecting unintended species. Hence, RNAi molecule/species specificity, dosage optimization, and delivery vehicle selection should be designed and verified for different purposes to mitigate off-target effects in aquaculture.

In contrast, early gene transfer was achieved by random integration of recombinant DNA into the target genome by microinjection or electroporation. For example, morphological alterations from GH gene transfer were observed in various fishes. Some P₁-generation GH-transgenic coho salmon showed excessive levels of GH, resulting in morphological abnormalities in the head, fin, jaw, and operculum [89]. WT rainbow trout possessing foreign GH not only had increases in growth rates, but also showed cranial abnormalities [320]. The possible hypotheses are that GH transgenesis affects growth pathways beyond those controlled by homeostatic processes that maintain normal morphology and viability [4], or that random integration of the foreign gene disrupts other functional genes due to off-target effects. CRISPR/Cas9-mediated genome editing/gene replacement induced low-probability off-target events in fish species. For example, lethal off-target effects on fry were observed when the *lh* locus was edited using ZFNs in channel catfish [112]. Furthermore, potential off-target events were presented when the *Hrg* transgene was inserted into chromosome 1 of channel catfish using a CRISPR/Cas9-mediated KI system [288]. Xing et al. [268] found that KI of the *eolv2* transgene at the *lh* locus of channel catfish resulted in morphological deformities caused by off-target effects. In particular, increased off-target events were observed in fish when double genes were integrated into targeted sites compared to single gene insertions [168, 274]. However, no obvious off-target effects were detected in some studies, even in the same fish species. No detectable off-target phenotypes were observed when the *GnRH* of channel catfish was mutated via TALEN-mediated KO [117], or when *Cath* was inserted into the non-coding regions of channel catfish via CRISPR/Cas9-mediated KI [77]. These studies showed that occurrence of off-target events depended upon the experimental design, target specificity, and sensitivity of detection methods. Indeed, sgRNA target sites can be predicted by conducting BLAST (basic local alignment and search tool) searches against the genome of the target species or by using other bioinformatic tools to optimize sgRNA design, such as Cas-OFFinder [321] and CRISPRdirect [322], to minimize off-target effects. In addition, high-fidelity Cas nucleases, such as Cas9 variants (e.g., CRISPR/Cas9-HITI, dCas9-SSAP), have been engineered to exhibit higher specificity by reducing off-target binding [323, 324].

Another obstacle to the application of genome manipulation in commercial aquaculture is mosaicism, especially in gene transfer and genome editing. Mosaicism is a common phenomenon observed in both transgenesis and genome editing techniques in aquaculture species and other organisms. Mosaicism occurs when an organism contains cells with different genetic compositions due to incomplete or variable incorporation of the introduced genetic material or edit during early development [4, 325], causing a low frequency of germline transmission of the foreign DNA or mutant sequences. In gene transfer, if the microinjected DNA fragment integrated after the one-cell embryonic stage, the animal would be mosaic, for the transgene could have contained one or multiple copies in some cells and none in others. Mosaic individuals have been detected in GH-transgenic channel catfish [84], Nile tilapia [326], common carp [87], coho salmon [89], and mud loach [327]. Similarly, genome-editing techniques such as CRISPR/Cas9 can lead to mosaicism if the editing machinery induces genetic modifications in only a portion of the targeted cells [325]. After CRISPR/Cas9-mediated DNA cleavage, the repair process can vary from cell to cell, resulting in the incorporation of edited or unedited DNA sequences. As a result, edited and unedited cells coexist within the organism, leading to mosaicism. Zhong et al. [115] reported that a *mstn*-mutant common carp had different types of indels in its genomic DNA, indicating potential mosaicism. In addition, high mosaicism was observed in *mstn*-edited red sea bream [161]. Recently, mosaic *eolv2*-transgenic channel catfish were identified by quantitative real-time PCR or intraspecific hybridization [78, 269]. Mosaic blue catfish were detected by *Cath* being expressed in only six of nine tissues examined when the *Cath* transgene was integrated into the *lh* locus [79]. Given the occurrence of off-target effects, it is essential to screen the tissues of the progeny for mosaicism to ensure that the genetic modifications are consistent and complete across all cells, thereby preventing unintended variability and ensuring the reliability of experimental outcomes. Nonetheless, a mosaic broodstock carrying transgenes or mutations in the germline can be used in traditional selective breeding to obtain multi-general transgenic or gene-edited progeny.

Another critical challenge in the genome manipulation of aquaculture species is the identification of causative genes responsible for economically important traits, such as growth, disease resistance, and reproductive performance. Currently, the number of known genes linked to these traits in aquaculture species is limited. This limitation hampers the ability to effectively apply genetic manipulation techniques, such as gene editing, to enhance these traits. While some gene functions are conserved across different species, including humans, the functions of many genes can vary significantly between species. This variation means that relying solely on knowledge derived from model organisms or other animals may not always lead to successful trait enhancement in aquaculture species. Consequently, a focused effort on identifying specific gene variants that directly influence key traits in aquaculture species is essential. The identification of these causative gene variants requires comprehensive genomic studies, including GWAS, QTL mapping, and functional genomics approaches. Once these genes are identified, targeted genetic manipulation can be employed to introduce beneficial variants or knock out deleterious ones, leading to more effective and predictable outcomes in aquaculture breeding programs.

4.2 | Government Regulation

Concern has been expressed about the possible ecological or genetic impacts of genetically modified fishes. Data to date indicates reduced fitness of transgenic fish compared to wild conspecifics, which is positive for biological and environmental risk containment [4]. GH-transgenic channel catfish fry are more susceptible to predation than WT counterparts under natural conditions without supplemental feeding in confined earthen ponds [328], and GH-transgenic coho salmon fry are more easily preyed upon than WT controls [329]. An alternative explanation could be starvation due to increased metabolism of the transgenic fry [226]. GH-transgenic first-feeding rainbow trout fry had lower survival in stream mesocosms in the presence of predators [330], possibly due to the additive effect of the transgene that negatively decreased their foraging ability and risk of predation [226]. The fitness of “all-fish” GH-transgenic common carp was lower than that of control fish in a 6.7-ha artificial lake mimicking carp habitat in the Yangtze River in China, as reflected by decreased swimming speed and viability [331]. Taken together, it appears that these transgenic fish are less fit than non-transgenic and WT individuals, and transgenes would likely be selected against in the natural environment [226], indicating little environmental risk from transgenic fish.

However, the ecological and genetic risks of releasing transgenic fish into the wild extend beyond immediate fitness disadvantages. Studies such as those by Devlin et al. [332] and Muir and Howard [333] highlight that even less-fit transgenic individuals can interbreed with wild populations, potentially introducing transgenes that could have deleterious effects upon ecosystem dynamics. These impacts may take generations for natural selection to address, underlining the importance of rigorous, long-term environmental risk assessments. This concept, known as the “Trojan gene hypothesis,” suggests that transgenes, even if they confer lower overall fitness, could spread through wild populations if they increase mating success, potentially leading to significant ecological consequences.

The food safety of GM fish has been assessed by testing the effects on the health of model mammals when fed with GM fish ingredients. Alimuddin et al. [334] found that Wistar rats exhibited no significant differences in behavior, histopathology, urea, or creatinine levels when fed a diet containing GH-transgenic common carp meal. Results from other studies show similarities. No biological differences were observed in clinical signs of toxicity, body weight, food intake, hematology, serum chemistry, and organ histopathology in GH-transgenic carp after a 90-day subchronic feeding study in Wistar rats [335]. Recently, the food safety of all-female gene-edited common carp was evaluated through a 90-day feeding trial. The findings indicated that there were no significant differences in body weight, FCR, hematology, serum biochemistry, gross necropsy, and histopathological examination compared to the control group when Wistar rats were fed a diet containing such transgenic fish meal [336]. In addition, the U.S. Food and Drug Administration (FDA) conducted a comprehensive evaluation of AquAdvantage Atlantic salmon, the first transgenic fish approved for human food use. The FDA determined that AquAdvantage salmon is as safe and nutritious as non-genetically engineered Atlantic salmon [337]. These studies have conducted a thorough safety assessment of

GM fish by recording and comparing various parameters, including but not limited to nutritional composition, allergenicity, toxicity, and unintended effects on fish physiology. By critically evaluating these aspects of food safety, stakeholders can make informed decisions regarding the safety of consuming farmed fish strains produced using transgenesis and genome editing in the future.

Extensive work has shown that genetically engineered aquatic animals have little ecological impact and are safe for food. The U.S. FDA strongly supports the use of genome editing and other genome manipulation tools to bring new, innovative products to market that can improve human and animal health, enhance animal well-being, and improve food production and quality [338]. Regulatory frameworks governing the release and commercialization of genetically engineered aquatic species are critical [339, 340], but they vary widely across jurisdictions, posing challenges for researchers, developers, and industry stakeholders seeking to navigate a patchwork of regulations. Therefore, harmonizing regulatory standards, conducting comprehensive risk assessments, and establishing transparent channels of communication [341] are critical steps in addressing regulatory challenges and fostering public confidence in the development and deployment of genetically engineered aquatic species.

In addition to regulatory challenges, consumer acceptance plays a key role in any future commercialization process. Society appears to be more accepting of genome editing than of transgenesis [340], because the small indel-induced mutation from genome editing maybe equivalent to naturally occurring allelic mutation. Indeed, consumers often prioritize safety when considering GM foods, but generally have a very low level of awareness of transgenesis and genome editing. Therefore, greater efforts should be devoted to educating the public about the science, benefits, and potential risks associated with these genome manipulation technologies [4, 340], which can help dispel misconceptions and foster acceptance. By addressing these considerations and engaging with consumers in a transparent and responsive manner, stakeholders can work towards fostering greater acceptance of GM aquaculture species in the marketplace.

5 | Prospects and Perspectives

5.1 | Multi-trait Enhancement

Previous studies have shown that enhancement of growth, disease resistance, fatty acid content, tolerance to low DO, or controllable reproduction can be achieved by editing a single endogenous gene or introducing a single exogenous gene. It is possible to improve multiple traits in parallel using CRISPR/Cas9-mediated genome editing and gene replacement in aquaculture, where targeted modification/insertion can achieve desired phenotypic outcomes. Using CRISPR/Cas9 or other genome-editing tools, researchers can simultaneously introduce edits to multiple genes targeting different traits within the same organism. This approach allows the simultaneous improvement of multiple traits in aquaculture species. Recently, transgenic channel catfish carrying *elovl2* gene and *mc4r* mutations showed faster growth and higher DHA levels than WT individuals [269]. Xing et al. [268] succeeded in KI of an *elovl2* transgene at the

lh locus, producing *elovl2*-transgenic channel catfish with improved n-3 fatty acid content and potential sterility. Moreover, to obtain transgenic fish lines with reversible sterilization and improved disease resistance, the alligator AMP gene *Cath* was integrated into the *lh* locus of channel catfish and blue catfish using ssODN or dsDNA-mediated CRISPR/Cas9 systems [79, 81]. Based on these empirical data and theoretical foundations, our hypothesis is that replacing the original functional genes with AMGs in specific coding regions of the chromosome would confer multi-generational antimicrobial activities to the host and improve multiple producer-favored traits. This strategy will hopefully allow us to create new fish lines possessing multiple traits, such as enhanced growth and disease resistance, sterilization and disease resistance, double enhanced disease resistance, improved DHA content and disease resistance, or hybrid lines with all of these improved traits (Figure 6). Although these studies demonstrated that simultaneous gene KI and KO might support more rapid attainment in reducing potential deleterious environmental risks of transgenic fish, the potential pleiotropic/off-target effects caused by multiple-gene editing/replacement should be considered. Nonetheless, it is important to note that the development of true-breeding lines and their subsequent crossing would be necessary to stabilize these traits, a process that requires significant time and financial investment. This would also need to be followed by rigorous regulatory approval processes, which add further complexity and cost. Given these challenges, such an ambitious breeding program would likely be feasible only for well-funded breeders or organizations with the resources to sustain long-term development and navigate the extensive regulatory landscape.

Given the slow progress in bringing biotech animals to market, it is important to carefully consider the realistic prospects for the commercialization of multi-modified animals. The development and approval processes for such animals are likely to be even more complex and time-consuming due to the need for extensive safety and environmental impact assessments, as well as the potential for greater public scrutiny.

5.2 | Novel CRISPR Platforms

CRISPR/Cas9 technology has revolutionized genome editing across various fields, including aquaculture. Researchers continue to refine existing tools and explore new approaches in the dynamic field of genome editing. The Cas9 enzyme was the primary focus of the initial stages of CRISPR development, while novel CRISPR platforms have emerged to enhance the precision, efficiency, and versatility of genome editing. Some of these novel CRISPR platforms may show promise for aquaculture applications. For example, CRISPR/Cas12a-mediated genome editing has been tested in vitro and in vivo, resulting in relatively high editing efficiencies in fish species. However, compared to Cas9, studies showed a lower editing efficiency for the *slc45a2* gene in Atlantic salmon (> 90% vs. 63%) and rainbow trout (> 90% vs. 67%) cell lines using the Cas12a enzyme [130]. López-Porras et al. [139] reported that use of the Cas9 enzyme resulted in a higher KO efficiency (31% vs. 11%) than Cas12a for targeting the *slc45a2* gene in Atlantic cod. Although CRISPR/Cas12a-mediated genome editing offers advantages in mammalian cells, lower efficiency than CRISPR/Cas9 was observed in fish

species, which may be due to differences in target recognition and PAM requirements. Cas9 recognizes a specific sequence of about 20 bp (the sgRNA), whereas Cas12a recognizes a longer sequence of about 23–24 bp. This longer recognition sequence might decrease the likelihood of off-target effects, where the Cas protein binds and cuts DNA at unintended sites, leading to reduced efficiency and potential unintended mutations. In addition, Cas9 typically requires a PAM sequence of NGG, which is relatively common in the genome. In contrast, Cas12a recognizes a T-rich PAM sequence, which may be less prevalent in fish genome, limiting the number of available target sites and reducing editing efficiency. Nevertheless, these studies have expanded the toolbox for genome editing in aquaculture.

As a recent advancement in genome editing, base editing allows for direct nucleotide changes without DSBs, providing a high-efficiency, simple, and universal strategy for engineering base substitutions at target sites. The cytosine base-editing (CBE) system, consisting of a cytidine deaminase fused with an nCas9 (D10A) and an uracil glycosylase inhibitor, converts targeted cytosine to uracil in genomic DNA [342]. In addition to CBE, adenine base editing (ABE), which mediates the conversion of A to G in the target DNA sequence, was also developed by David Liu's group [343]. Both base editors can induce premature stop codons to produce loss-of-function proteins and have been applied in zebrafish with high efficiency of on-target single-base substitutions [344, 345]. With respect to farmed fishes, base editors can be easily designed for in vitro and in vivo applications. To date, CBE is the only base-editing method that has been applied to genome editing in Atlantic salmon, with findings showing an average of 50%–66% correct conversion of the target base [142]. Compared to Cas9-induced multiple indels, CBE or ABE preserves the reading frame of the gene and potentially reduces the risk of unintended consequences, and this artificially mediated point mutation is closer to the result of natural random mutation.

Epigenome editing tools, such as CRISPR/dCas9-mediated CRISPRi (CRISPR interference) and CRISPRa (CRISPR activation), enable targeted gene regulation without altering DNA sequences [346]. These technologies could revolutionize genetic research, biotechnology, and medical therapies by providing unprecedented control over genetic information. As RNA-guided genomic transcriptional regulation tools, CRISPRi and CRISPRa consist of a catalytically dead Cas9 (dCas9), a transcriptional effector and a customizable sgRNA. This type of dCas9/sgRNA complex is incapable of cleaving DNA, but retains the ability to specifically bind to target DNA, resulting in transcriptional regulation similar to RNAi but with different mechanisms [347]. Extensive work is currently being done in crops to enhance the resistance or tolerance to biotic and abiotic stresses using CRISPR/dCas9 systems [348]. In contrast, the lack of CRISPR/dCas9-mediated epigenome editing was documented in fish species. Fukushima et al. [349] reported an approach using dCas9 combined with sgRNA to induce repressive histone modification in a locus-specific manner in medaka. Obviously, the study of epigenetics in fish species is still relatively nascent compared to that in mammals or plants. The lack of comprehensive epigenomic data and understanding of epigenetic regulatory mechanisms in fishes may hinder the development and application of CRISPR/dCas9-mediated epigenome-editing tools.

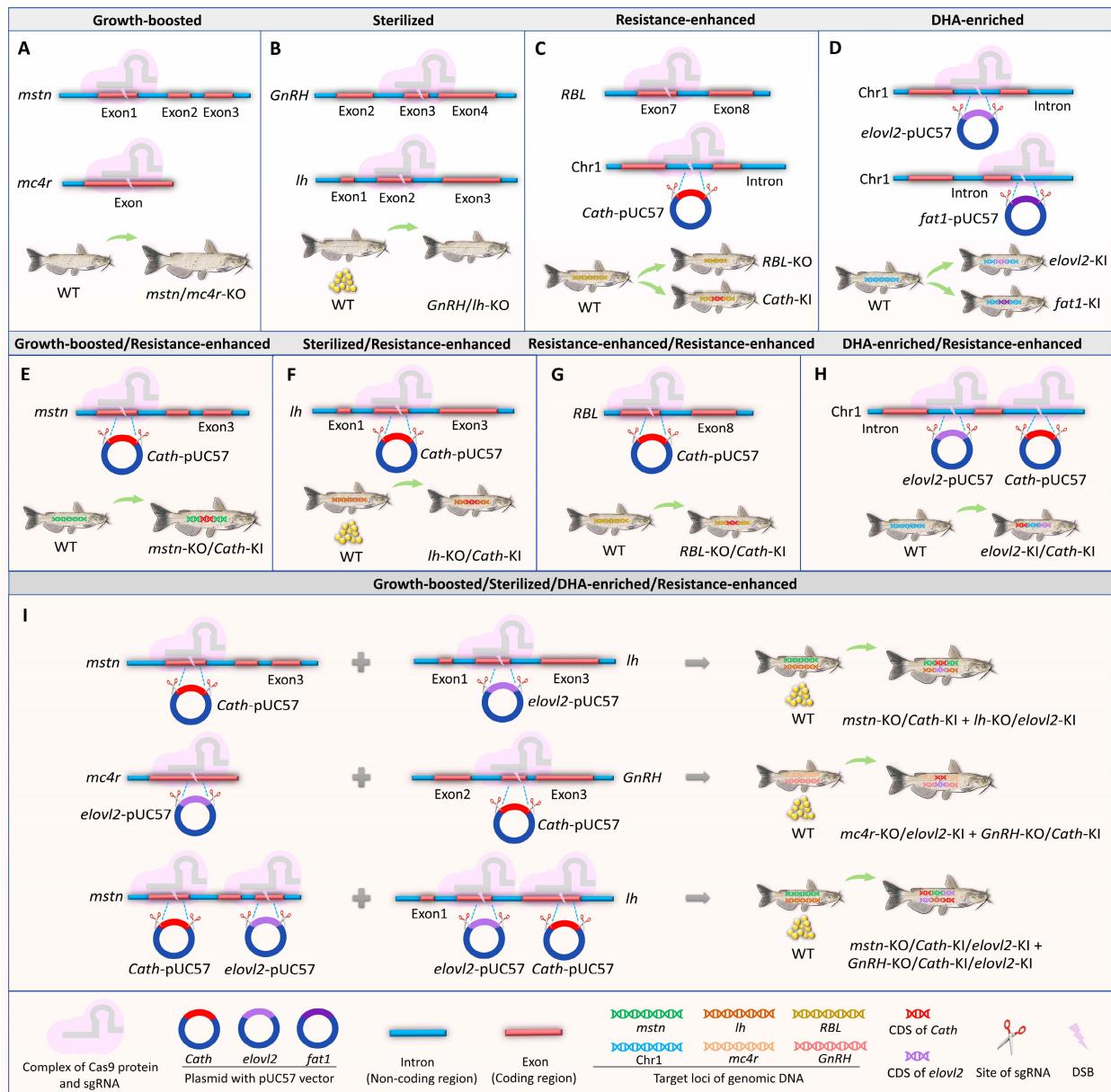


FIGURE 6 | Designed CRISPR/Cas9-mediated transgenesis induces traits of interest to disease resistance enhancement combined with sterile, growth-boosted, and DHA-enriched traits in channel catfish (*Ictalurus punctatus*) and other fish species. (A) Growth-boosted fish genetic lines were created by knocking out the *mstn* or *mc4r* gene. (B) Sterile fish lines were produced via knocking out the *GnRH* or *lh* gene. (C) Disease resistance-enhanced fish lines were created by knocking out *RBL* or knocking in the cathelicidin (*Cath*) transgene at the non-coding region of chromosome 1. (D) DHA-enriched fish line was generated through knocking in the *elovl2* or *fat1* gene at the non-coding region of chromosome 1. (E) Disease resistance-enhanced fish with fast-growth was produced by knocking in the *Cath* gene at the *mstn* locus. (F) Disease resistance-enhanced fish with sterility were produced by knocking in the *Cath* gene at the *lh* locus. (G) A higher disease resistance-enhanced fish line was created by knocking in the *Cath* gene at the *RBL* locus. (H) Disease resistance-enhanced fish with high DHA content was produced by knocking in the *Cath* and *elovl2* genes at the non-coding region of chromosome 1. (I) Multiple CRISPR/Cas9 systems produce fish lines that contain enhanced-disease resistance, fast-growth, sterility, and enriched-DHA traits. DHA, docosahexaenoic acid; *RBL*, like rhamnose-binding lectin; *elovl2*, elongase of very-long-chain fatty acids 2; *fat1*, humanized omega-3 desaturase, encodes a desaturase enzyme that converts n-6 to n-3 fatty acids; *mstn*, myostatin; *mc4r*, melanocortin 4 receptor; *GnRH*, gonadotropin-releasing hormone; *lh*, luteinizing hormone; Chr1, chromosome 1; KO, knock out; KI, knock in; *Cath*–*elovl2*–*fat1*-pUC57, a plasmid containing the CDS of *Cath*/*elovl2*/*fat1* genes constructed with pUC57 as the vector; CDS, coding sequences; DSB, double-stranded break.

To reduce off-target mutations, efforts have been made in the field of basic biology or basic medicine via new variants of the Cas9-derived systems (*Easi*-CRISPR, C-CRISPR, CRISPR/Cas9-HITI and dCas9-SSAP) [323–325], but these need further improvements for non-medical therapeutic applications. Compared to CRISPR/Cas9 or CBE/ABE system, these advanced tools need

to be chemically modified to activate or generate fusion of Cas9 variants, which requires researchers in the biological field to have a foundation in synthetic biology or chemistry. Therefore, it may be technically challenging and require significant research and development efforts to design and optimize such novel CRISPR systems for specific gene modification in fish.

6 | Conclusions

Three leading genome manipulation approaches, including gene transfer, RNAi and genome editing, have been reviewed in this paper. As the aquaculture industry continues to navigate the complexities of genome manipulation, this review provides a synthesis of the current knowledge base and points to the potential transformative impact of genome manipulation on the future of aquaculture breeding.

Meanwhile, the advent of genome manipulation technologies in aquaculture has ushered in a new era of opportunities to improve the traits of aquatic species. The precision of these tools allows the development of lines with enhanced performance, marking the potential for a significant shift in the traditional breeding programs used in the industry. Genome manipulation represents a cutting-edge approach in aquaculture that holds great promise for addressing key challenges and opening new possibilities for sustainable and high-performance aquatic food production. As the field continues to advance, the responsible integration of genome manipulation into aquaculture practices holds the potential to revolutionize the industry, ensuring its resilience, environmental responsibility, and contribution to global food security.

Author Contributions

Jinhai Wang: writing – original draft. **Yu Cheng:** writing – original draft. **Bao Feng Su:** writing – review and editing. **Rex A. Dunham:** writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.