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Review article

Genes for editing to improve economic traits in aquaculture fish species

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ABSTRACT

Aquaculture, a critical sector for global food security, faces the challenge of meeting growing demand while protecting wild fish populations. Gene editing, a powerful genetic tool, emerges as a potential solution. By modifying key genes in fish, it is expected to rapidly enhance growth rate, disease resistance, and other economically important traits, leading to increased profitability, sustainability, and competitiveness of the industry. This review navigates the complex landscape of genes for important traits, gene editing in aquaculture, exploring successes, challenges, and prospects for improved productivity, sustainability, and resilience. We provide guidance for researchers and stakeholders in identifying and editing genes responsible for important traits, while addressing economic, environmental, regulatory, and ethical considerations. This roadmap paves the way for a future where gene editing empowers aquaculture to meet global food needs while upholding environmental responsibility.

1. Introduction

Aquaculture has emerged as a pivotal industry in global food production, addressing the increasing demand for aquatic products while relieving the pressures on dwindling wild fish stocks (FAO, 2022). Aquaculture is a varied field with around 600 fish species raised, according to the United Nations' Food and Agriculture Organization (FAO). But, only 20–30 of these species play a major role in global production (FAO, 2022; Yue, Tay, et al., 2024). However, as the aquaculture sector expands to meet the needs of a growing human population, it faces multiple challenges ranging from environmental sustainability to production efficiency (Naylor, et al., 2021). To address these challenges, the application of genetic tools, specifically gene editing (Blix, et al., 2021; Hallerman et al., 2023; Luo et al., 2022; Yang, Yu, Tay, & Yue, 2022), has garnered substantial attention in recent years.

Gene editing, with its precision and versatility, offers the potential to revolutionize aquaculture by facilitating the targeted modification of specific genes responsible for economic traits such as growth rate, disease resistance, and feed efficiency (Barman, et al., 2017; Blix et al., 2021; Hallerman et al., 2023; Luo et al., 2022; Yang, Yu, Tay, & Yue, 2022). Unlike traditional selective breeding (Gjedrem, et al., 2012; Yue, Wang, et al., 2024), which relies on natural variation and often entails protracted breeding cycles, gene editing allows for rapid and deliberate genetic improvements (Yang, Yu, Tay, & Yue, 2022). This transformative technology promises to enhance the profitability, environmental sustainability, and overall competitiveness of the aquaculture industry.

The quest to harness the full potential of gene editing in aquaculture species is a complex endeavour (Blix, et al., 2021; Hallerman et al., 2023; Yang, Yu, Tay, & Yue, 2022). It necessitates an in-depth exploration of the genetic factors that underlie the traits of interest (Houston, et al., 2020; Yue, 2014), the development and optimization of editing techniques, and the careful assessment of both economic and environmental implications. Moreover, it demands a comprehensive understanding of the regulatory landscape and the ethical considerations surrounding the application of gene editing in aquatic organisms (Hallerman, et al., 2023; Yang, Yu, Tay, & Yue, 2022).

This review explores known genes for economically important traits, gene editing's diverse applications in aquaculture, examining edited genes and their economic impact on the aquaculture industry, as well as approaches for identifying genes for important traits for gene editing. The paper meticulously examines the accomplishments, challenges, and prospects associated with gene editing, aiming to enhance the productivity, sustainability, and resilience of aquaculture species. Additionally, it briefly discusses the economic and environmental assessments, regulatory frameworks, and ethical dilemmas that accompany gene editing in aquaculture. Through a thorough analysis of key genes and genetic mechanisms influencing economic traits, this review highlights gene editing's transformative potential, emphasizing the im-

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portance of responsible and sustainable implementation. Our objective is to provide a comprehensive overview of genes for editing to accelerate genetic improvement of important traits for researchers, policymakers, and industry stakeholders to optimize the benefits of this revolutionary technology for a more efficient and environmentally conscious aquaculture industry.

2. Economic traits in aquaculture

Several key traits significantly impact the success of aquaculture operations, including growth rate, disease resistance, feed efficiency, reproductive traits, meat quality, and adaptation to climate change (Fig. 1), each with its unique genetic underpinnings (Gjedrem, et al., 2012; Yue, 2014). Growth rate is a valuable economic trait in aquaculture, directly influencing production efficiency (Coogan, et al., 2022b; Kim et al., 2019a; Zhang et al., 2020). Disease resistance is another vital aspect of aquaculture sustainability. Fish and shellfish are susceptible to various pathogens, which can lead to significant economic losses (Elaswad and Dunham, 2018; Houston, 2017). Feed efficiency is a crucial trait for optimizing resource utilization in aquaculture. Certain genes and genetic elements influence nutrient utilization and metabolic efficiency (Huang, et al., 2021; Yang et al., 2023b). Reproductive traits, such as spawning frequency and reproductive success, are essential for maintaining breeding populations (Xu, et al., 2023; Zohar, 2021). Skin colour is often a heritable trait of economic importance. Some genes influencing skin colour are known (Jiang, et al., 2022; Luo et al., 2021; Xu et al., 2019). Omega-3 content in aquaculture species is influenced by various factors, including diet and genetic factors. Specific genes are typically associated with omega-3 production in fish (Datsomor, Zic, et al., 2019; Lin et al., 2018; Xing, Su, Li, et al., 2022; Xing, Su, Bangs, et al., 2022; Young, 2009). In addition, omega-3 levels are often attributed to dietary sources of these essential fatty acids (Tocher, 2003). The existence of intermuscular bones in certain fish species poses challenges for human consumption(Nie, et al., 2020), potentially diminishing the market demand for fish with a higher prevalence of such bones. (Li, et al., 2021a; Nie et al., 2020). This situation can influence the economics of both fishing and aquaculture industries, affecting the availability of specific fish species for human consumption.(Mubango, et al., 2023). Using gene editing (Blix & Myhr, 2023; Hallerman, 2021; Hallerman et al., 2023; Yang, Yu, Tay, & Yue, 2022) to reduce or eliminate intramuscular bone is an ideal method for addressing this issue (Dong, et al., 2023; Gan et al., 2023; Kuang et al., 2023). The ability to adapt to climate change is crucial for aquaculture species because it helps them thrive in changing environmental conditions (Galappaththi, et al., 2020). Rising temperatures, changes in water chemistry, and extreme weather events can negatively impact aquaculture systems. Species capable of adapting to these changes are more likely to survive, reproduce, and maintain stable production levels, thereby ensuring food security and economic sustainability amidst a changing climate.

In conclusion, key factors for advancing aquaculture sustainability and profitability include growth rate, disease resistance, feed efficiency, omega-3 content, reproductive traits, appearance, and adaptability to climate change. Leveraging selective breeding (Gjedrem, et al., 2012; Houston et al., 2020; Mubango et al., 2023; Yue, 2014), molecular breeding [10,11], and gene editing (Gjedrem, et al., 2012; Houston et al., 2020; Mubango et al., 2023; Yanez et al., 2022; Yue, 2014) can lead to production of more resilient, efficient, and productive fish stocks, reducing environmental impacts and ensuring a stable supply of high-quality seafood for a growing global population. Ongoing research may reveal additional genes associated with economic traits, and the understanding of these genetic mechanisms is continually evolving. Combining gene editing, molecular breeding and selective breeding further enhances control and optimization of these traits in aquaculture species. Researchers in the field of aquaculture genetics are continuously identifying and manipulating genes to improve economic traits for sustainable and efficient aquaculture production.

3. Gene editing techniques

Gene editing has revolutionized the field of aquaculture breeding by providing precise tools to manipulate the genetic makeup of aquaculture fish species (Barman, et al., 2017; Blix & Myhr, 2023; Hallerman, 2021; Hallerman et al., 2023; Yang, Yu, Tay, & Yue, 2022). Three prominent gene editing techniques utilized in aquaculture are Zinc finger nucleases (ZFNs) (Urnov, et al., 2010), Transcription activator-like effector nucleases (TALENs) (Sanjana, et al., 2012), and clustered regularly interspaced short palindromic repeats-Cas 9 (CRISPR/Cas9) (Jinek, et al., 2012). These techniques possess unique capabilities and applications for improving economic traits and overall sustainability in aquaculture operations. Here, we provide a brief introduction to these three approaches, as detailed information on the gene editing steps and related issues in aquaculture are available in several comprehensive review papers (Blix, et al., 2021; Ferdous et al., 2022; Gratacap et al., 2019; Hallerman et al., 2023; Houston et al., 2020; Yang, Yu, Tay, & Yue, 2022).

ZFNs are an older gene editing method (Urnov, et al., 2010) but have been used successfully in aquaculture applications. ZFNs enable targeted gene modifications. Researchers have employed ZFNs to en-

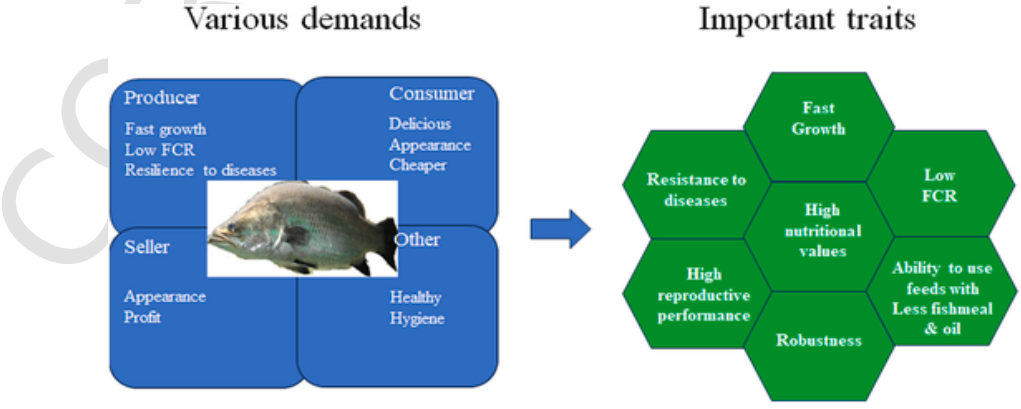

Fig. 1. Key economic traits of fish in the aquaculture sector, categorized according to diverse requirements.
The primary demands for economic traits in aquaculture fish species originate from aquaculture producers, fish product sellers, seafood consumers, and other factors, including considerations for health and hygiene (left). On the right, the major economic traits include growth, disease resistance, low feed conversion ratio (FCR), high nutritional value, strong reproductive performance, resilience to climate change, and the ability to thrive on feeds with reduced fishmeal and fish oil.

hance traits such as growth and disease resistance in some aquaculture species (Dong, et al., 2011). However, ZFNs require the design of custom protein-DNA binding domains, which can be complex and costly.

TALENs are another gene editing technology (Sanjana, et al., 2012) that has found application in aquaculture. TALENs offer precise genome editing by introducing double-strand breaks in the DNA at specific target sites. This technique has been employed to modify genes related to disease resistance in fish species like Nile tilapia (*Oreochromis niloticus*) (Li, et al., 2013). TALENs are often considered less user-friendly and more resource-intensive.

CRISPR/Cas9, a versatile and widely adopted gene editing tool, allows for precise modification of an organism's DNA (Fig. 2). In aquaculture, CRISPR/Cas9 has been used to target genes associated with growth rates, disease resistance, and other economically valuable traits. Recently, new CRISPR-Cas systems, including CRISPR/Cas12/Cas13/Cas14 proteins have been developed for gene editing purposes (Hillary and Ceasar, 2023). For experiments and design of guide RNA (gRNA), readers may refer to reviews (Liu, Zhang, & Zhang, 2020; Luo et al., 2022). CRISPR-Cas systems stand out as highly effective gene-editing tools, utilizing a unique RNA-based system, in contrast to ZFNs and TALENs, which rely on polypeptide-DNA binding (Jinek, et al., 2012). ZFNs involve peptide binding to a DNA triplet, whereas TALENs utilize short peptides binding to a DNA nucleotide. Unlike CRISPR-Cas, both ZFN and TALEN require dual target molecules on the complementary strand, introducing greater complexity to cloning and expanding the target sequence area for greater specificity. However, careful selection of target sequence in CRISPR-Cas can mitigate this. The advantages of CRISPR-Cas lie in its universal applicability across various organisms, simplicity in reagent creation, and the availability of affordable commercial CRISPR products. Selection of the appropriate duide RNA (gRNA) is crucial, taking into account sequence similarity and proximity to a protospacer adjacent motif (PAM) sequence to reduce off-targets effects (Pickar-Oliver & Gersbach, 2019). Preassembled ribonucleoprotein (RNP) complexes can further reduce off-target effects. CRISPR-Cas utilizes target-specific gRNAs and Cas enzymes to induce double-strand breaks, requiring a specific PAM for each Cas protein. While some Cas proteins have limitations, such as NGG requirements, advances in identifying new Cas proteins or synthetic evolution overcome these limitations (Hillary and Ceasar, 2023). The versatility of CRISPR-Cas extends to DNA binding properties, allowing for gene activation, gene function repression, genomic region visualization, and epigenetic modification. This adaptability showcases the universal utility of this technology.

4. Edited genes for economic traits, achievements, and issues in aquaculture species

Gene editing has been applied to some aquaculture species to improve traits such as growth rate (Coogan, et al., 2022b; Coogan et al., 2022b), Disease resistance (Yang, Yu, Wang, et al., 2022; Yang, Yu, Tay, & Yue, 2022), and adaptability to diets with lower fishmeal (Huang, et al., 2021). Different species have been targeted using various gene editing techniques, with a focus on specific genes or genetic elements associated with economically significant traits. Here are some examples of genes that have been targeted in gene editing (Table 1) for aquaculture species.

4.1. Edited genes for growth traits

Myostatin, also known as GDF8, is a protein encoded by the *mstn* gene in humans (Dong, et al., 2011; McPherron and Lee, 1997; Tao et al., 2021; Wu et al., 2023a). It is a myokine produced by muscle cells and inhibits muscle growth. Myostatin belongs to the TGF beta protein family and is produced in skeletal muscle before entering the bloodstream. Studies show that inhibiting myostatin results in increased muscle mass (McPherron and Lee, 1997). Individuals with mutations in both copies of the myostatin gene have significantly more muscle and strength. In 1997, geneticists Se-Jin Lee and Alexandra McPherron identified the myostatin gene by creating mice without it, resulting in a significant increase in muscle (McPherron and Lee, 1997). This led to them being dubbed mighty mice. Natural deficiencies in myostatin have been observed in some cattle, sheep, whippets, and humans, all leading to a notable increase in muscle mass (Aiello, et al., 2018; Lee, 2004). Editing of *mstn* has been reported in many aquaculture species, including yellow catfish (*Pelteobagrus fulvidraco*) (Dong, et al., 2011; Zhang et al., 2020), common carp (*Cyprinus carpio*) (Zhong, et al., 2016), red sea bream (*Pagrus major*) (Kishimoto, et al., 2018), olive flounder (*Paralichthys olivaceus*) (Kim, et al., 2019a), Pacific oyster (*Crassostrea gigas*), blunt snout bream (*Megalobrama amblycephala*) (Sun, Zheng, et al., 2020), Nile tilapia (Li, et al., 2021c; Wu et al., 2023a), mud loath (*Misgurnus anguillicaudatus*) (Tao, et al., 2021), channel catfish (*Ictalurus punctatus*) (Coogan, et al., 2022b), large yellow croaker (*Larimichthys crocea*) (Yan, et al., 2022), and blotched

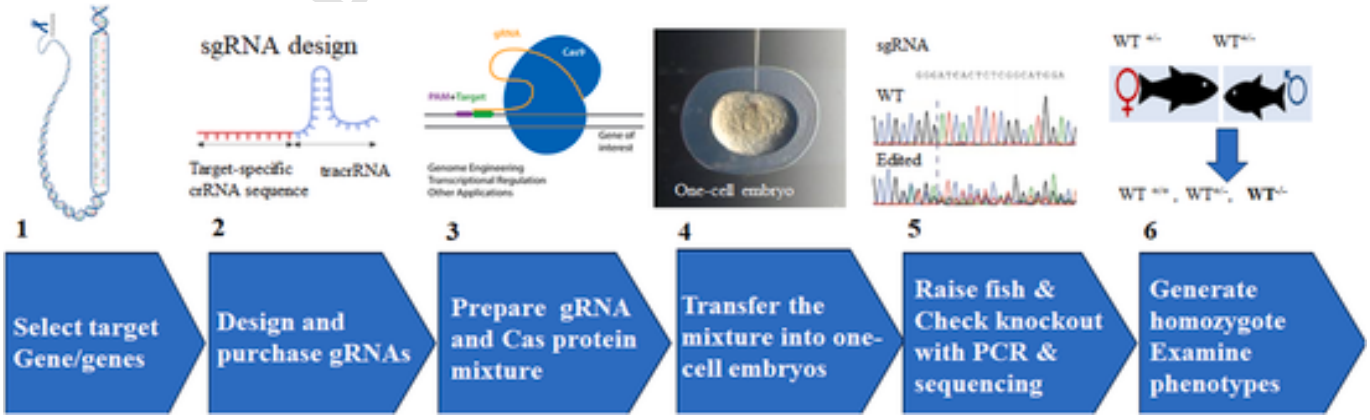

Fig. 2. Key steps (1–6) in gene editing in Aquaculture fish species.
sgRNA: single guide RNA; crRNA: crisprRNA, which a 17–20 nucleotide sequence complementary to the target DNA and a tracrRNA, which serves as a binding scaffold for the Cas nuclease; gRNA: a guide RNA, which is a piece of RNA that functions as a guide for RNA- or DNA-targeting enzymes, with which it forms complexes; PAM: protospacer adjacent motif. This short, conserved sequence of 2–5 bp is located next to target DNA and required to discriminate between 'self' and 'non-self'. PAM motifs are not present near spacers of the CRISPR locus to avoid autoimmunity and cleavage of the host genome; WT: wild type; Edited: mutant generated with gene editing; $WT^{+/-}$: Knockout heterozygote; and $WT^{-/-}$: Knockout homozygote.

Table 1
A list of major edited genes for important traits in aquaculture species.

Traits	Genes	Species	References
Growth	*mstn*	*Elteobagrus fulvidraco*, *Cyprinus carpio*, *Pagrus major*, *Paralichthys olivaceus*, *Crassostrea gigas*, *Megalobrama amblycephala*, *Oreochromis niloticus*, *Misgurnus anguillicaudatus*, *Ictalurus punctatus*, *Larimichthys crocea*, *Channa maculata*	(Coogan, et al., 2022b; Dong et al., 2011; Kim et al., 2019a; Kishimoto et al., 2018; Li et al., 2021b; Ou et al., 2023; Sun, Zheng, et al., 2020; Tao et al., 2021; Wu et al., 2023b; Yan et al., 2022; Yu, Li, et al., 2019; Zhang et al., 2020; Zhong et al., 2016)
	epr	*Takifugu rubripes*	NEWs (2022)
	mc4r	*Ictalurus punctatus*	Coogan, et al. (2023)
	pomc	*Danio rerio*	(Wan, et al., 2021; Yang et al., 2023a)
	pi3k	*Carassius gibelio*	Huang, et al. (2021)
	1r1	*Danio rerio*	Cai, et al. (2021)
	igfbp	*Oncorhynchus mykiss*	Cleveland, et al. (2018)
Intramuscular bones	*runx2*,	*Danio rerio*	(Dong, et al., 2023; Gan et al., 2023; Nie et al., 2022)
	bmp6	*Carassius auratus*	Kuang, et al. (2023)
	wist	*Oryzias latipes*	Yasutake, et al. (2004)
	sp7	*Cyprinus carpio*	Zhong, et al. (2016)
Reproduction	*lh*	*Ictalurus punctatus*	Qin, et al. (2016)
	gsdf	*Oryzias latipes*	(Jiang, et al., 2016; Zhang et al., 2016)
	wt1	*Oreochromis niloticus*,	Jiang, et al. (2017)
	nanos2, *nanos3*, *dmrt1*, *foxl2*	*Oreochromis niloticus*	Chen, et al. (2017)
	eef1a	*Oreochromis niloticus*	Chen, et al. (2017)
	piwil2	*Oreochromis niloticus*	Jin, et al. (2020)
	pfpdz1	*Pelteobagrus fulvidraco*	Dan, et al. (2018)
	dnd	*Salmo salar* L., *Oncorhynchus mykiss*	(Fujihara, et al., 2022; Wargelius et al., 2016)
	cyp17a1	*Cyprinus carpio* L., *Monopterus albus*	(Hou, et al., 2023; Zhai et al., 2022)
	dmrt1	*Cynoglossus semilaevis*, *Betta splendens*	(Cui, et al., 2017; Wang, Sun, et al., 2022)
	cyp19a1a and *foxl2*	*Monopterus albus*	Hou, et al. (2023)
	esrs	*Oreochromis niloticus*	Yan, et al. (2019)
	amhy	*Oreochromis niloticus*	Li, et al. (2015)
	amhr2	*Oreochromis niloticus*	Liu, Xiao, et al. (2020)
	vasa	*Oreochromis niloticus*	Li, et al. (2019)
Disease resistance	*rrm1*	*Danio rerio*	Yang, et al. (2021)
	gab3	*Danio rerio*	Yang, Yu, Wang, et al. (2022)
	viperin	*Carassius gibelio*	Mou, et al. (2022)
	camp	*Ictalurus furcatus*	Agier, et al. (2015)
	cec and *cath*	*Ictalurus punctatus*	Wang, Su, et al. (2023)
	itgb1b	*Gobiocypris rarus*	Chen, et al. (2018)
	tlr22	*Labeo rohita*	Chakrapani, et al. (2016)
Meat quality	*fads2*	*Salmo salar* L	Datsomor, Olsen, et al. (2019)
	elovl2	*Salmo salar* L., *Ictalurus punctatus*	(Datsomor, Zic, et al., 2019; Xing, Su, Bangs, et al., 2022)
	fat-1 and *fat-2*	*Ictalurus punctatus*	Xing, et al. (2023)
Pigmentation	*tyr*	*Salmo salar* L., *Carassius carassius*, *Oryzias latipes*, *Paramisgurnus dabryanus*,	(Edvardsen, et al., 2014; Fang et al., 2018; Liu et al., 2019; Xu et al., 2019),
	slc45a2	*Salmo salar*	Straume, et al. (2020)
	scarb1 and *scarb1-like*	*Cyprinus carpio*	Du, et al. (2021)
	pmela and pmelb, *pmel-17*	*Oreochromis niloticus*, *Oreochromis mossambicus*	(Liu, et al., 2022a; Liu et al., 2022b; Sun et al., 2024; Wang, Xu, et al., 2022; Wang, Sun, et al., 2023)
	lrp2aB	*Carassius auratus*	Yu, et al. (2022)
	bco2	*Exopalaemon carinicauda*	Sun, Liu, et al. (2020)
	mitf	*Oreochromis niloticus*	Wang, Kocher, et al. (2023)
	hps4	*Oreochromis niloticus*	Wang, Kocher, et al. (2022)
	asip	*Cyprinus carpio* var. *color*	Chen, et al. (2019)
	mc1r	*Cyprinus carpio* var. *color*	Mandal, et al. (2020)
Other traits			
Accept plant protein	*t1r1*	*Danio rerio*	Huang, et al. (2021)
Chitinase function	*ecchi4*	*Exopalaemon carinicauda*	Gui, et al. (2016)
Behaviour	*secretogranin-2a and -2b*	*Danio rerio*	Mitchell, et al. (2020)
Development-related	*scxa*,	*Danio rerio*	(Nie, et al., 2021),
Ammonia resistance	*chop*	*Isgurnus anguillicaudatus*	Lv, et al. (2023)

snakehead (*Channa maculata*) (Ou, et al., 2023). The first myostatin-edited fish, the yellow catfish, was created using ZFN technology in 2011 (Dong, et al., 2011). Subsequently, all myostatin-edited fish have been generated with the CRISPR/Cas systems. In all the *mstn*-edited fish species, there were observed improvements in growth performance. In the red sea bream, editing of *mstn* with CRISPR/Cas9 resulted in a 16 % increase of skeletal muscle mass and a reduced body length (Kishimoto, et al., 2018). In Nile tilapia, a study found that *mstnb* ($^{-/-}$) fish exhibited notably better growth performance parameters. After 5 months post hatch in a laboratory setting, these fish exhibited a 1.99 times higher weight gain rate, a 1.77 times higher condition factor, and a 1.23 times higher specific growth rate compared to *mstnb* ($^{+/+}$) fish (Wu, et al., 2023a). The knockout of the *mstn* gene through CRISPR/Cas9 technology enhances growth and disease resistance in channel catfish (Coogan, et al., 2022b). In the red sea bream, knockout of the *mstn* gene enabled fish to grow approximately 1.2 times larger while consuming the same amount of food (Kishimoto, et al., 2018). Hence, the *mstn* gene stands out as the most frequently targeted gene for genetic modification in aquaculture species. In Japan, two *mstn*-edited fish species, the tiger puffer (*Takifugu rubripes*) and the red sea bream, have received approval for sale (NEWs, 2022).

The leptin receptor, discovered in 1995 (Tartaglia, et al., 1995), is encoded by the *lepr* gene and belongs to the type I cytokine receptor family. It functions as the receptor for the hormone leptin, which is specific to fat cells (Tartaglia, et al., 1995), and responsible for appetite regulation. Japanese scientists successfully knocked out the *lepr* genes in tiger pufferfish. Removal of four *lepr* genes resulted in increased food consumption and accelerated weight gain. Consequently, the modified fish achieve a weight 1.9 times greater than traditional tiger pufferfish, enabling them to reach market size more rapidly (Kishimoto, et al., 2019).

Melanocortin 4 receptor (*mc4r*) is a melanocortin receptor that is encoded by the *mc4r* gene in humans (Magenis, et al., 1994). MC4 receptors have been found to be involved in feeding behaviour, the regulation of metabolism, sexual behaviour, and male erectile function (Huszar, et al., 1997). In 2017, researchers used three sgRNAs and the Cas9 protein to target the *mc4r* gene in channel catfish embryos, achieving a 33% mutation rate, with 33% of the fish becoming homozygous or bi-allelic for the mutation. About 71% of the offspring inherited this

mutation, resulting in enhanced growth compared to control fish across various stages and environments. In earthen ponds, homozygous/biallelic mutants reached market size 30% faster than heterozygous mutants. By the stocker stage (around 50 g) in 2019, the *mc4r* × *mc4r* mutants were 40% larger than the combined CNTRL × CNTRL families and 54% larger than F_1 *mc4r* × CNTRL mutants, indicating a recessive trait associated with the mutation. The high mutation rate, inheritance, and improved growth make gene-edited *mc4r* channel catfish advantageous for commercial farming (Coogan, et al., 2022a).

The *pomc* gene plays a crucial role in controlling feeding behavior, sexual differences, growth, and pigmentation (Wan, et al., 2021). The utilization of *pomc* knockout zebrafish allowed for an investigation into the impact of *pomc*-knockout on factors such as survival, growth, feed conversion ratio (FCR), tissue histology, liver histology, and the expression of genes linked to fatty acid and cholesterol synthesis (Shi, et al., 2020; Yang et al., 2023b). When compared to WT and *pomc* $^{\pm}$ zebrafish, *pomc*$^{-/-}$ zebrafish exhibited increased body weight and reduced FCR. Histological analysis revealed an increased production of skeletal muscle without any signs of obesity in *pomc*-knockout zebrafish. *Pomc* knockout did not affect the histology of the brain, liver, or the survival rate. The expression of genes associated with fatty acid and cholesterol synthesis suggested that *pomc* mutants could potentially lower cholesterol levels. These findings demonstrate that *pomc* knockout enhances growth performance and reduces FCR in zebrafish without adverse effects on other important traits. Consequently, *pomc* represents a valuable gene for genetic modification using CRISPR/Cas systems to enhance growth and reduce FCR in aquaculture species (Yang, et al., 2023b).

Phosphatidylinositol 3-kinase (*pi3k*) signalling plays an indispensable role in vertebrate metabolism and energy homeostasis (Denver, et al., 2011). In Gibel carp (*Carassius gibelio*), the edited fish showed improvement in somatic growth and feed conversion efficiency (Huang, et al., 2021). Disruption of the *pik3r1* gene in Gibel carp enhanced PI3K/AKT/mTOR signalling, leading to notable benefits. Unlike in mammals, this disruption did not significantly affect glucose levels but resulted in reduced triglycerides, amino acids, and lactate. Importantly, it significantly improved somatic growth, lipid utilization, and feed conversion efficiency in the mutant fish.

The T1R receptors are a family of taste specific C G protein-coupled receptors that consists of three proteins, namely T1R1, T1R2, and T1R3, which are encoded by their corresponding genes, *tas1r1*, *tas1r2*, and *tas1r3* (Treesukosol, et al., 2011). The T1R G-protein-coupled receptors for sweet taste have been shown to have multiple binding sites, used by sugars, artificial sweeteners, and sweet taste antagonists (Roper, 2007). A recent study in China established that deletion of the *t1r1* gene significantly improved growth performance and acceptance of plant proteins in zebrafish (Cai, et al., 2021). Therefore, editing *t1r1* in fish aquaculture species may increase the capacity to use feeds with more plant-based proteins.

While successful gene editing for growth in fish holds great promise, most studies have predominantly focused on growth, neglecting analysis, and reporting of data on other crucial economic traits such as FCR, meat quality, disease resistance, and adaptability to diverse environments. Recognizing this knowledge gap, it is imperative to conduct comprehensive experiments that thoroughly examine these aspects before releasing gene-edited fish for commercial production. This approach will ensure a complete understanding of the potential benefits and implications of gene editing in fish farming, making the future of this technology even more exciting and promising.

4.2. Edited genes for eradiating or reducing intermuscular bones (IBs)

Gene editing has shown promising results in reducing or eliminating IBs in certain fish species, such as common carp, blunt snout bream, amphitriploid gibel carp (*Carassius gibelio*), and crucian carp (*Carassius auratus*) (Dong, et al., 2023; Gan et al., 2023; Kuang et al., 2023). Various candidate genes, including *bmp6* and *runx2*, associated with the development of these bones have been utilized in these studies (Dong, et al., 2023; Kuang et al., 2023). In this section, we provide an overview of the findings and address pertinent concerns relating to the removal of these small bones in aquaculture settings.

Related transcription factor 2 (*runx2*), encoded by the *runx2* gene in humans, is a vital transcription factor in osteoblast differentiation (Lucero, et al., 2013). It also seems to regulate cell proliferation, especially in osteoblasts and endothelial cells by influencing the G1 phase of the cell cycle. The levels of *runx2*'s peak in G1 and decrease in S, G2, and M phases (Van Der Deen, et al., 2012). Although the full extent of *runx2*'s cell cycle regulation is not entirely understood, it's recognized that its activity levels impact cell cycle entry, exit, and progression. These functions are crucial, particularly in bone cancer development such as osteosarcoma, where aberrant cell proliferation control plays a role (Li, et al., 2021a; Nie et al., 2020). Inhibition of *runx2*'s DNA-binding activity hinders osteoblastic differentiation, earning it the title of the "master regulator of bone". This gene has been knocked out in some aquaculture species to reduce intermuscular bones. In zebrafish, *runx2b*$^{-/-}$ mutants exhibited a complete loss of IBs, however, swimming performance, growth and bone mineral density were not significantly different from *runx2b*$^{+/+}$ zebrafish (Nie, et al., 2022). Generation of intermuscular bone-free mutants in amphitriploid gibel carp was achieved through the editing of two duplicated *runx2b* homeologs (Dong, et al., 2023; Gan et al., 2023). Blunt snout bream without IBs were created by mutation of the *runx2b* gene (Dong, et al., 2023). In all these reported studies, knockout of the *runx2b* gene resulted in the reduction or complete removal of IBs. However, these studies did not provide information on whether the knockout of *runx2b* affects other commercially important traits, such as disease susceptibility, adaptability to environment changes, meat quality, and other economic advantages. Mutation of the *runx2b* gene in humans is linked to many diseases. Specifically, mutations in the *runx2* gene are associated with cleidocranial dysplasia (CCD), which is a rare congenital disorder that affects the development of bones and teeth (Otto, et al., 2002). Variants of *runx2* have been associated with the osteosarcoma phenotype (San Martin, et al., 2009). Therefore, a thorough examination of the effects of *runx2b* knockout in aquaculture species is essential for a comprehensive understanding of the potential consequences.

The bone morphogenetic protein 6 (*bmp6*) gene is a gene found in humans and encodes a protein belonging to the bone morphogenetic protein (BMP) family. BMPs are a group of signalling proteins that play crucial roles in various aspects of embryonic development, tissue regeneration, and homeostasis, with a particular emphasis on bone and cartilage formation. *Bmp6*, in particular, is associated with several important functions in the body (Hahn, et al., 1992). Disruption of the *bmp6* gene in zebrafish leads to the absence of IBs, with no discernible impact on their growth and reproductive capabilities (Xu, et al., 2022). Knockout of the *bmp6* gene in crucian carp results in the elimination of IBs (Kuang, et al., 2023). However, it is worth noting that disruption of various parts of the *bmp* signalling pathway in mouse models resulted in either severe deformities or death of the embryos (Wang, et al., 2014), highlighting the important role of the pathway in embryonic development. This indicates the need for caution when considering the potential negative effects of modifying the IBs on economically valuable traits in aquaculture species. It is crucial to conduct thorough evaluations to ensure that any modifications do not unintentionally impact the overall health or desirable characteristics of these fish species in aquaculture settings.

The bHLH transcription factor Twist (*twist*), within the Twist family, plays a pivotal role in guiding the differentiation of mesenchymal cells into osteoblasts (Marofi, et al., 2019). In barbel steed (*Hemibarbus labeo*), the transcriptomes of *twist1* and *twist2* exhibit significant changes across the four stages of IB development. In medaka (*Oryzias*

latipes) with *twist1b*-knockdown, certain vertebrae positioned bilaterally at the anterior end of the centra exhibit an absence of neural arches (Yasutake, et al., 2004). Thus, the *twist* gene may be a candidate gene to reduce IBs.

Transcription factor Sp7 (*sp7*, also called Osterix, *osx*) serves as a specific transcription factor in osteoblasts, activating genes that guide preosteoblasts through differentiation into mature osteoblasts and osteocytes. It has a dual role in intramembranous ossification, as it not only promotes osteoblast differentiation but also hinders chondrocyte formation (Sinha & Zhou, 2013). In common carp, CRISPR/Cas9-induced *sp7*a-knockout mutants display a reduced and smaller phenotype in comparison to wild-type controls. These mutants manifest severe bone development defects, such as inadequate opercula and maxilla, curved spines, deformed centrums, irregular hemal spines, and shorter intervertebral bones (IBs) (Zhong, et al., 2016). The bone abnormalities observed in *sp7a* mutants significantly surpass those seen in CRISPR-Cas9-*sp7b* mutants. Both sp*7a* and *sp7b* mutants display delayed development of craniofacial and centrum bones in comparison to wild-type controls (Zhong, et al., 2016). Additional abnormalities in *sp7a* mutants include smaller, irregularly shaped scales, and a reduced number of pharyngeal teeth. Therefore, this gene might not be a suitable candidate for eradicating or reducing IBs as its knockout negatively affects development.

The use of gene editing for the reduction or removal of IBs in aquaculture species is an area of active research and experimentation. Although there has been some promising research, it is essential to conduct additional experiments and field tests to examine whether eradicating IBs will affect other economically important traits, such as the swimming ability, feeding, disease resistance and overall health of the fish.

4.3. Edited genes for sex and reproductive sterility

Fish sex in aquaculture is crucial for aquaculture production, which enhances desirable traits like growth rate, disease resistance, and size (Li, et al., 2022). Understanding fish sex and reproduction aids in managing population dynamics and optimizing feeding and environmental conditions for each gender, ultimately improving aquaculture efficiency and production (Martínez, et al., 2014). For instance, in tilapia aquaculture, the efficiency of all-male production surpasses that of mixed-sex production (Bardhan, et al., 2021). Achieving reproductive sterility presents several possibilities for reducing potential environmental impacts stemming from aquaculture (Xu, et al., 2023). Animals raised in aquaculture settings can occasionally escape, and this may have consequences for the surrounding ecosystems. For instance, if a non-native species can pose ecological problems if it establishes a self-sustaining population or has detrimental genetic effects on populations it can interbreed with (Jensen, et al., 2010). Reproductive sterility also safeguards the interests of breeders who have made investments in improving the genetics of a particular farmed variety (Xu, et al., 2023). Moreover, inhibiting premature maturation in farmed fish contributes to enhanced fish welfare and improved growth performance (Li, et al., 2022; Martínez et al., 2014). Therefore, research has focused on achieving reproductive sterility in important species, utilizing genome editing techniques to induce sterility in Nile tilapia, channel catfish, Atlantic salmon, and other fish species.

The first gene editing in fish reproduction targeted the luteinizing hormone (*lh*) gene. This modification, generated using ZFN, was reported in channel catfish in 2016 (Qin, et al., 2016). Using a modified zinc finger nuclease technology with electroporation, *lh* was edited to induce sterility in channel catfish, resulting in the development of sterile individuals (Qin, et al., 2016).

In medaka, the gonadal somatic cell derived factor (*gsdf*) gene was deleted using ZFN technology, resulting in homozygous mutant fish displaying an all-female phenotype and reduced expression of the double-sex and mab-3 related transcription factor (*dmrt1*) (Zhang, et al., 2016). A similar outcome was observed in tilapia (Jiang, et al., 2016). Using CRISPR/Cas9, the tilapia *gsdf* gene was disrupted, leading to male-to-female sex reversal with ovotestis in the F_0 mosaic XY fish. The homozygous *gsdf* mutants all exhibited a female phenotype and had notably higher estradiol levels compared to the control fish (XY $gsdf^{+/+}$ and XY $gsdf^{+/-}$). These findings suggest that *gsdf* might play a role in inhibiting estrogen production in the gonads (Jiang, et al., 2016).

In Nile tilapia, the urinary and genital systems have a close relationship during embryonic development. An essential factor for urogenital system development is Wilms tumour 1 (*wt1*). In teleost fishes, there are two *wt1* genes, *wt1a* and *wt1b* (Jiang, et al., 2017). When both *wt1a* and *wt1b* genes were knocked out in Nile tilapia, distinct effects were observed (Jiang, et al., 2017). The $wt1a^{-/-}$ fish displayed pericardial edema and yolk-sac edema at 3 days after hatching (dahs), which later progressed to general body edema by 6 dahs. These fish failed to develop the glomerulus and typically did not survive beyond 10 dahs. In contrast, the $wt1b^{-/-}$ fish exhibited normal physical characteristics. Immunohistochemical analyses at 6 dahs showed the presence of the germ-cell marker vasa in $wt1a^{-/-}$ gonads, while somatic genes such as *cyp19a1a*, anti-Müllerian hormone (*amh)*, *gsdf*, and *dmrt1* were not expressed. The sex phenotypes of XX and XY in $wt1b^{-/-}$ fish remained unaffected. However, XX $wt1b^{-/-}$ fish displayed an up-regulation of ovarian *cyp19a1a* expression and higher levels of serum estradiol-17β at 90- and 180-days post hatch. In contrast, XY $wt1b^{-/-}$ fish maintained normal serum estradiol-17β and 11-ketotestosterone levels and remained fertile. Additionally, the knockout of the *amh* gene confirmed that a missense SNP on the Y chromosome is crucial for male sex determination in this species (Li, et al., 2015). A study was conducted using the CRISPR/Cas9 system to disrupt selected genes, such as Nanos C2HC-Type Zinc Finger 2 (*nanos2*), Nanos C2HC-Type Zinc Finger 3 (*nanos3*), *dmrt1*, and Forkhead box protein L2 (*foxl2*) (Li, et al., 2014). The study showed that mutations induced by CRISPR/Cas9 in *dmrt1* and *foxl2* were effectively passed down to the F_1 generation through the germline. In the F_0 generation, clear and noticeable phenotypes were observed following the mutation of genes specific to either germ cells or somatic cells. When *nanos2* and *nanos3* were lost in XY and XX tilapia, it resulted in gonads deficient in germ cells. In the case of the eukaryotic elongation factor 1 alpha (*eef1a*), which plays a crucial role in the translation process, the CRISPR/Cas9-mediated mutation of *eef1a1b* resulted in spermatogenesis arrest and infertility in the F_0 XY Nile tilapia (Chen, et al., 2017). Consistently, when the *eef1a1b* gene was heterozygously mutated ($eef1a1b^{+/-}$), it led to the absence of spermatocytes at 90 dah, with very few spermatocytes, spermatids, and spermatozoa observed at 180 dah. Additionally, there was a decrease in *cyp11b2* expression and serum 11-ketotestosterone levels at both stages. The fertility of $eef1a1b^{+/-}$ XY tilapia was compromised due to abnormal spermiogenesis. Transcriptomic analysis of the $eef1a1b^{+/-}$ testis from 180 dah XY fish revealed significant down-regulation of key factors involved in spermatogenesis, steroidogenesis, and sperm motility compared to control XY tilapia. Transgenic overexpression of *eef1a1b* rescued the spermatogenesis arrest phenotype seen in $eef1a1b^{+/-}$ testes. Another gene of interest, *piwil2*, is specific to gonads and maternally deposited in Nile tilapia eggs. Its function involves the repression of transposable elements, making it crucial for preserving germline cell fate. More precisely, the PIWI domain, a functional segment within *piwil2*, was the target of Cas9 mRNA and sgRNAs injected into Nile tilapia embryos at the one-cell stage. The outcomes revealed that 54% of the mutant larvae subjected to injection exhibited either a complete absence or a reduced number of putative primordial germ cells (PGCs) compared to control fish (Jin, et al., 2020). The *tgf-β* signalling pathway plays a crucial role in gonadal sex determination. The *amhy* gene is identified as the key factor inducing male sex determination. Located on the Y chromosome, *amhy* differs from the X-linked *amh* due to a missense SNP, resulting in an amino acid change. *Amhy* lacks a promoter sequence found

in the X-linked *amh*. Knockout of *amhy* in XY fish leads to male-to-female sex reversal, while *amh*Δ-y mutation alone does not (Li, et al., 2015). Heterozygous mutations in *amh* or *amhr2* increased primary growth follicles and decreased fertility, while homozygous mutations resulted in hypertrophic ovaries, increased primary follicles, and a failure in transitioning to vitellogenic follicles (Liu, Xiao, et al., 2020). Deleting the *vasa* 3′ UTR lowered *vasa* mRNA expression in the gonad (Li, et al., 2019). Estrogen, acting through estrogen receptors (ESRs), is vital for regulating reproductive activities in vertebrates. In a study (Yan, et al., 2019), mutant lines for *esr1*, *esr2a*, and *esr2b* were generated in tilapia using CRISPR/Cas9. Among them, *esr2a* mutant females showed a delay in ovarian development, while *esr2a* mutant males demonstrated smaller gonads and abnormal sperm. The *esr2b* mutants had infertility issues due to abnormal development of reproductive ducts.

In yellow catfish, a newly discovered gene (*pfpdz1*) containing a PDZ domain plays a critical role in male sex differentiation and maintenance (Dan, et al., 2018). The coding sequence of *pfpdz1* on the Y chromosome is virtually identical to that on the X chromosome, except for a missense SNP (A/T) that results in an amino acid change (E to V) in the N-terminal region. *Pfpdz1* demonstrates male-specific expression during the process of sex differentiation. When *pfpdz1* is overexpressed through additive transgenesis, individuals develop testis-like tissue instead of ovaries. Conversely, targeted inactivation of *pfpdz1* on the Y chromosome using CRISPR/Cas9-mediated mutagenesis resulted in ovarian differentiation (Dan, et al., 2018).

In Atlantic salmon, the dead end (*dnd*) gene plays a vital role in germ cell development, which forms the sperm and eggs in various animals, including humans. It functions in germ cell formation, migration, and survival, while also regulating gene expression and preventing them from becoming somatic cells (Youngren, et al., 2005). Mutations in *dnd* can cause reproductive issues (Baloch, et al., 2021). Recent research involving *dnd* knockout in salmon has potential implications for aquaculture. Germ cell-free (GCF), sterile salmon lacking *dnd*, showed no significant differences in body size, smoltification markers, or stress indicators compared to wild-type (WT) salmon (Wargelius, et al., 2016). Towards harvest size, WT salmon had higher growth rates, condition factors, and liver sizes, likely due to puberty initiation. GCF salmon's advantage lies in their ability to remain in a non-mature state, allowing for delayed harvest and thereby improving growth potential (Kleppe, et al., 2022). This research suggests the potential for using GCF salmon in aquaculture to address maturation and genetic introgression issues. In addition, in rainbow trout (***Oncorhynchus mykiss***), CRISPR/Cas9 was used to generate homozygous *dnd* gene mutants. The resulting *dnd* KO mutants, showed a decline in primordial germ cells (PGCs) over time, disappearing completely by one year. When used as recipients for germ cell transplantation, *dnd* KO rainbow trout successfully nurtured donor-derived germ cells, producing only donor-derived sperm and eggs in numbers equivalent to wild-type fish. These findings highlight the suitability of *dnd* KO rainbow trout as recipient candidates with a high capacity for supporting donor-derived germ cells (Fujihara, et al., 2022).

In common carp, the creation of an exclusively female population was achieved through the utilization of neomale carp with Cytochrome P450 17A1 (*cyp17a1*) deficiency (Zhai, et al., 2022). *Cyp17a1*$^{+/-}$ XX females displayed an average body weight 6.60% higher (at 8 months post-fertilization) and 32.66% higher (at 12 months post-fertilization) than that of the control common carp.

In Chinese tongue sole (*Cynoglossus semilaevis*), knockout of *dmrt1* in genetic males led to phenotypic females, which demonstrates the significant impact of genome editing in establishing *dmrt1* as a crucial male sex-determining gene (Cui, et al., 2017). In fighting fish (*Betta splendens*), the use of the CRISPR/Cas9 system to knock out *dmrt1* revealed that the absence of *dmrt1* function leads to female development (Wang, Sun, et al., 2022).

In the rice field eel (*Monopterus albus*), a hermaphroditic fish, multilocus gene editing successfully disrupted both *cyp19a1a* and *foxl2* (Hou, et al., 2023). In mutants with a disrupted *cyp19a1a* gene, there was a notable decrease in serum estrogen levels, leading to a halt in ovarian development. In the gonads of 12-month-old *cyp19a1a* mutant fish, only germ stem cells or oogonia were found, suggesting that *cyp19a1a* plays a pivotal role in regulating the initial stages of gonadal development within the ovaries of rice field eels. Conversely, in *foxl2* mutants, while there was a minimal decrease in *cyp19a1a* transcription and serum estrogen levels, there was an increase in the transcription of *foxl2-l*, *foxl3*, and *dmrt1*. Surprisingly, despite these changes, the gonads of *foxl2* mutant fish still differentiated into ovaries. This indicates that *foxl2* does not appear to directly influence ovarian development in rice field eels.

In aquaculture, gene editing techniques like CRISPR/Cas9 are being used to manipulate the sex and reproductive sterility of species. Researchers are exploring methods to control the sex of fish, a valuable trait for species with size or gender-based market preferences. This can enhance production efficiency and reduce environmental impact. Additionally, gene editing can be used to create sterile fish to prevent unwanted breeding in aquaculture systems, mitigating concerns about escapees and genetic interactions with wild populations.

4.4. Edited genes for disease resistance

CRISPR/Cas9 and other gene editing technologies present promising avenues for improving disease resistance in aquaculture species (Yang, et al., 2020, 2021). Through precise DNA modifications, researchers can enhance the immune responses of these species and reduce susceptibility to various diseases.

Viral nervous necrosis (VNN), caused by nervous necrosis virus (NNV), poses a significant economic threat to aquaculture. Recent research in Asian seabass identified a key locus for NNV resistance on linkage group 20 (Liu, Wang, Wong, & Yue, 2016; Liu, Wang, Wan, et al., 2016; Wang et al., 2017). An association study involving 445 survivors and 608 mortalities following NNV challenge highlighted a significant correlation between a SNP in the *rrm1* gene and NNV resistance. In humans, the *rrm1* gene encodes the enzyme, ribonucleoside-diphosphate reductase large subunit (Parker, et al., 1995). The enzyme is essential for the production of deoxyribonucleotides prior to DNA synthesis in the S phase of dividing cells (Parker, et al., 1995). In Asian seabass, functional analysis confirmed that over-expression of *rrm1* reduced viral RNA and titer in infected cells, while its knock-down increased viral replication. Zebrafish with a *rrm1* knockout showed heightened susceptibility to NNV infection. These findings implicate *rrm1* as a pivotal gene for NNV resistance (Yang, et al., 2021).

GAB3-GRB2-associated binding protein 3 (*gab3*), a member of the GRB2-associated binding protein gene family, serves as a scaffolding protein in growth factor and cytokine signalling pathways (Wolf, et al., 2002). It harbours a pleckstrin homology domain, interacting with SHP2 tyrosine phosphatase and GRB2 adapter protein. GAB3 aids in macrophage differentiation through alternative splicing, yielding multiple transcript variants (Wolf, et al., 2002). A previous study on GWAS for NNV resistance showed that the *gab3* gene plays a pivotal role in NNV resistance in Asian seabass (Wolf, et al., 2002; Yang et al., 2020). Knocking down *gab3* in NNV-exposed seabass cells significantly reduced viral RNA and titers. In zebrafish, *gab3* knockout enhanced survival and resistance against NNV. Thus, silencing *gab3* emerges as an effective strategy to inhibit NNV replication (Yang, Yu, Wang, et al., 2022).

Viperin is increasingly recognized as a key protein in antiviral response. In hexaploid gibel carp (*Carassius gibelio*), there are two duplicated viperin homeologs: *cgviperin*-A and -B (Mou, et al., 2022). The depletion of *cgviperin*-A using the CRISPR/Cas9 technique primarily impacted immune-related pathways, while the removal of *cgviperin*-B in-

fluenced pathways associated with autophagy. Intriguingly, simultaneous disruption of both genes resulted in the complete loss of gibel carp's resistance against CaHV. Notably, the absence of *cgviperin*-A had a notable impact on the expression of *cgviperin*-B in the CgViperin-A($^{-/-/-}$) mutant. In contrast, *cgviperin*-A transcripts remained highly expressed in the CgViperin-B$^{-/-/-}$ mutant, similar to the wild-type. These findings contribute to the understanding of viperin's antiviral activities.

Cathelicidin antimicrobial peptide (CAMP) is a protein found mainly in the lysosomes of certain immune cells, like macrophages and polymorphonuclear leukocytes (PMNs) (Agier, et al., 2015). In humans, the CAMP gene produces a larger protein called CAP-18 (18 kDa), which is transformed into its active form LL-37 through an external process involving proteinase 3-mediated cleavage (Agier, et al., 2015). One study demonstrated the successful integration of the alligator cathelicidin (As-Cath) gene using two CRISPR/Cas9-assisted knock-in (KI) systems in blue catfish (*Ictalurus furcatus*) (Agier, et al., 2015). The integration efficiency was high for both strategies, with 16.67% using linear double-stranded DNA (dsDNA) and 24.53% using a double-cut plasmid. The on-target KI efficiency of the double-cut plasmid strategy was 1.67 times higher than that of the linear dsDNA strategy. The transgenic blue catfish exhibited elevated expression of the *as-cath* gene in various tissues, including the kidney, skin, and muscle. Importantly, the transgenic fish showed significantly improved survival rates (80%) compared to wild-type controls (30%) following fish pathogen *Flavobacterium covae* infection, indicating enhanced disease resistance. The transgenic individuals also exhibited higher survival rates during culture. There were no discernible differences in growth rates or external morphology between transgenic and wild-type siblings, suggesting no negative impacts on those traits. Overall, this study demonstrates the potential of CRISPR/Cas9-assisted KI to enhance disease resistance in aquaculture species like blue catfish, offering a promising strategy for disease reduction.

In another study, CRISPR/Cas9-assisted microinjection was used to integrate cecropin (*Cec*) and cathelicidin (*Cath*) antimicrobial peptide genes (AMGs) into the fish genome, creating dual-AMG integrated (*_*Cec*$^{+}$/*_Cath^{+}) transgenic embryos with high efficiency. Following bacterial challenge, both single-AMG and dual-AMG integrated fish showed significantly higher survival rates than wild-type fish. Logistic regression analysis revealed that body weight and gender did not affect survival, while transgenes *Cec* and *Cath* directly contributed to survival during bacterial infection. This study demonstrated the feasibility of generating highly efficient dual-gene integrated genetic lines through CRISPR/Cas9-mediated genome editing and highlighted the promising role of transgenes in enhancing fish disease resistance (Wang, Su, et al., 2023). However, the knockin of important genes from other species using the CRIPSR/Cas9 technology is classified as genetically modified organisms (GMOs). Concerns persist in many countries regarding GMOs, including potential environmental impacts, unintended consequences, and corporate control of seeds. To address these concerns, rigorous safety assessments, transparent labelling, and regulations can help ensure responsible GMO development. Additionally, promoting independent research, encouraging diverse farming practices, and fostering public engagement in decision-making can help mitigate potential risks associated with GMOs.

In the rare minnow, *Gobiocypris rarus*, integrin β-1 (*itgb1b*) is a transmembrane protein crucial for viral entry. Using the CRISPR/Cas9 system, fishes with *itgb1b*-deficient were generated in the rare minnows (*itgb1b*$^{-/-}$) to explore *tgb1b*'s role in grass carp reovirus (GCRV) infection (Chen, et al., 2018). Upon GCRV stimulation, *itgb1b*$^{-/-}$ minnows showed extended survival compared to wild-type minnows. The *itgb1b*$^{-/-}$ group exhibited significantly lower GCRV copy numbers and reduced expression of clathrin-mediated endocytosis and apoptosis-related genes. These observations indicate that the lack of *itgb1b* diminishes the efficiency of viral entry and the expression of genes associated with apoptosis. This underscores its essential role in virus-cell entry via clathrin.

In *Labeo rohita*, the CRISPR/Cas9 system was used to knock out the Toll-like receptors (*tlrs*), which are major players for innate immunity (Chakrapani, et al., 2016). The generated null mutant exhibited a lack of TLR22 mRNA expression. However, it is not known yet whether the disruption of Toll-like receptor 22 (*tlr22*) gene resulted in any deficiency disease resistance.

The protein SECRETOGRANIN-2, found in secretory granules, is converted into the bioactive neuropeptide *secretoneurin*. SECRETOGRANIN-2 plays vital roles in controlling fertility (Mitchell, et al., 2020). In zebrafish, mutations in the *secretogranin-2a* and *-2b* genes result in disrupted sexual behaviours, decreased ovulation and egg laying, and reduced fertility and embryo survival (Mitchell, et al., 2020). This study suggests that modification of *secretogranin-2a* and *-2b* genes using CRIPSR/Cas systems may improve spawning in cultured fish species.

In general, edited genes for disease resistance in aquaculture species have the potential to significantly improve survival rates and productivity of aquaculture while reducing dependence on antibiotics and other treatments. Currently, only a few genes associated with disease resistance have been targeted successfully. Future research directions include identifying causative genes for disease resistance using linkage mapping (Yue, 2014) and GWAS (Wang, et al., 2021), as well as refining gene editing tools for specific aquaculture species (Yang, Yu, Tay, & Yue, 2022).

4.5. Edited genes for meat quality

Meat quality, especially omega-3 fatty acid levels, plays a vital role in human health (Young, 2009). Scientists have been editing specific genes involved in fatty acid synthesis to increase the omega-3 content in certain fish species. This section offers an overview of key findings from notable studies (Datsomor, Zic, et al., 2019; Xing, Su, Li, et al., 2022; Xing, Su, Bangs, et al., 2022; Xing et al., 2023) in this area, contributing to the understanding of the advancements in improving the beneficial omega-3 levels in fish meat.

One research study aimed to explore the roles and redundancy of Atlantic salmon fatty acyl desaturase (*fads2*) genes in the production of long-chain polyunsaturated fatty acids (LC-PUFAs) (Datsomor, Olsen, et al., 2019). Two groups of CRISPR-mediated partially modified salmon were generated: Δ6abc/5 Mt (with mutations in Δ*6fads2-a,* Δ*6fads2-b,* Δ*6fads2-c*, and Δ*5fads2*) and Δ*6bcMt* (with mutations in Δ*6fads2-b* and Δ*6fads2-c*). These fish were fed with feed containing varying compositions of LC-PUFAs. The results indicated that Δ*6abc*/5 Mt salmon had impaired synthesis of 22:6n-3, along with an accumulation of its precursors. In contrast, Δ6bcMt had no significant impact on Δ6-desaturation. Both groups accumulated substrates for Δ8-desaturation when fed a low LC-PUFA diet. In addition, Δ*6abc*/5 Mt exhibited an upregulation of the lipogenic transcription regulator, suggesting a connection between endogenous and dietary LC-PUFA synthesis in salmon. These findings provide new insights into the functions of salmon *fads2* genes and their influence on LC-PUFA synthesis. Therefore, editing *fads2* genes has the potential to modify the fatty acid profile of aquaculture fish species.

In another study, researchers explored the molecular mechanisms governing polyunsaturated fatty acids (PUFAs) synthesis in Atlantic salmon and their regulatory processes. Through CRISPR-mediated partial knockout of *elovl2*, a key enzyme in PUFA synthesis, they identified *elovl2* as essential for the synthesis of docosahexaenoic acid (22:6n-3) in multiple tissues (Datsomor, Zic, et al., 2019). The knockout salmon had diminished levels of 22:6n-3, along with an accumulation of other polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (20:5n-3) and docosapentaenoic acid (22:5n-3).

A study sought to augment the nutritional profile of channel catfish by incorporating a transgene from the masu salmon to increase omega-

3 fatty acid content (Xing, Su, Li, et al., 2022). CRISPR/Cas9-mediated knock-in targeted a non-coding region of chromosome 1, achieving a 19% integration rate compared to the 27.3% rate with random integration methods. Despite the lower integration rate, the CRISPR/Cas9 approach resulted in higher rates of transgene presence across multiple tissues. Genomic qRT-PCR indicated higher transgene copy numbers in CRISPR/Cas9 transgenic fish. Furthermore, CRISPR/Cas9 P1 fish exhibited stronger transgene expression and 20.7% higher levels of Docosahexaenoic acid (DHA) compared to controls, while random integration did not show similar effects. These findings highlight the efficiency and improved outcomes of CRISPR/Cas9 technology in transgenesis, enabling expedited breeding and enhanced gene function studies. Nonetheless, it's important to note that the gene introduction through gene editing techniques is considered genetic modification. In many countries, fish produced via gene knock-in using these methods are still classified as GMOs. Another study focused on enhancing n-3 polyunsaturated fatty acid (PUFA) levels in channel catfish through genetic modification (Xing, et al., 2023). CRISPR/Cas9 technology was used to insert *Caenorhabditis elegans fat-1* and *fat-2* genes, which encode n-3 fatty acid desaturases, into the catfish genome (Xing, et al., 2023). Precise insertions occurred at multiple genomic sites with a 9.1% on-target knock-in efficiency for *fat-1*, driven mainly by the HDR repair pathway. Transgenic fish expressed *fat-1* and *fat-2* genes, significantly increasing the levels of beneficial DHA and total n-3 fatty acids while reducing harmful n-6 fatty acids. These modifications offer potential benefits in enhancing fish nutritional value, reducing reliance on fish oil in aquaculture feed, and providing an alternative source of essential n-3 PUFAs.

Gene editing is able to increase the omega-3 content in aquaculture species, paving the way to improve fish with inherently low and suboptimal omega-3 levels for human health, such as freshwater Nile tilapia (Lin, et al., 2018; Young, 2009). Improving the omega-3 to omeg-6 ratio in the meat of freshwater Nile tilapia could be an interesting research field.

4.6. Edited genes for pigmentation

Pigmentation in animals is a complex process involving various molecular pathways that regulate the production, distribution, and maintenance of pigments responsible for the coloration of tissues (Luo, et al., 2021). The two main types of pigments in animals are melanin and carotenoids, and their synthesis and distribution are controlled by distinct molecular mechanisms (Hearing, 2011; Matsuno, 2001).

The melanin production pathway is a complex biochemical process responsible for the synthesis and distribution of melanin in the human body (Hearing, 2011). This pathway consists of several key stages, including tyrosine metabolism, melanogenesis, melanosome formation, melanocyte migration and distribution (Hearing, 2011). Melanin production begins with the amino acid tyrosine. The enzyme tyrosinase (Liu, et al., 2019) plays a crucial role in converting tyrosine into L-DOPA, a key precursor for melanin synthesis. L-DOPA undergoes a series of enzymatic reactions, involving tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2) (Kobayashi, et al., 1994) leading to melanin formation. These enzymes work together to convert L-DOPA into melanin, the pigment responsible for skin, hair, and eye color. Melanin synthesis takes place within specialized organelles known as melanosomes (D'Alba & Shawkey, 2019). Melanocytes, specialized pigment-producing cells, are responsible for producing these melanosomes. The intricate biochemical reactions required for melanin production occur in a controlled environment within these organelles. During embryonic development, melanocytes migrate from their place of origin in the neural crest to various parts of the body (Lapedriza, et al., 2014). This migration is essential for the even distribution of melanin throughout the skin and other tissues. The arrangement of melanin granules within skin cells determines the pattern and intensity of pigmentation in different regions of the body. Carotenoid accumulation is a biochemical process related to the acquisition and utilization of carotenoid pigments in animals (Sefc, et al., 2014). It encompasses several key stages: dietary intake and absorption, and transport, deposition, and metabolism. Animals obtain carotenoids from their diet, primarily through the consumption of fruits, vegetables, and various other food sources that contain these colourful compounds (Matsuno, 2001). After ingestion, carotenoids are absorbed from the digestive tract into the bloodstream. They are then transported to various tissues throughout the body. Carotenoids are deposited in tissues and organelles, such as chromoplasts, where they contribute to coloration. The presence of carotenoids in these structures can result in the vibrant hues seen in animals (Matsuno, 2001). Carotenoids are subject to enzymatic modifications within the body (Brush, 1990). These modifications can lead to the production of a diverse range of pigments.

Regulatory pathways are involved in the development of various pigments. The regulation of pigmentation in animals involves various mechanisms and factors including hormonal control, genetic regulation, environmental factors, and structural coloration (D'Mello, et al., 2016). Hormones, such as melanocyte-stimulating hormone (MSH) and signalling through the melanocortin-1 receptor (MC1R), play a pivotal role in controlling melanin synthesis and distribution (Herraiz, et al., 2021). These hormones can influence the intensity and pattern of pigmentation. Genes exert significant control over pigmentation pathways. They dictate the expression of enzymes and proteins involved in pigmentation. Genetic variations can lead to differences in coloration among individuals within a species (Sturm, et al., 2001). External environmental factors, including exposure to UV radiation, temperature, and nutrition, can have a profound impact on pigmentation. These factors can influence the availability of pigments, their utilization, and the overall coloration of an animal (Costin & Hearing, 2007). In some cases, animals achieve coloration not via pigments but through structural features. Examples include butterflies and peacocks, which possess microscopic structures in their tissues that interact with light, resulting in vibrant and iridescent colors (Kinoshita, et al., 2008). Structural coloration is a remarkable adaptation that enhances an animal's visual display without relying on pigments. In summary, the carotenoid pathway describes how animals obtain, absorb, transport, and utilize carotenoid pigments, while regulatory pathways encompass the various mechanisms and factors that control and influence pigmentation in animals, including hormonal, genetic, and environmental factors, as well as structural adaptations for coloration. These molecular pathways collectively determine the pigmentation patterns seen in various animal species. The specific mechanisms and genes involved can vary widely among different animals, leading to the diverse and fascinating array of colors and patterns observed in the animal kingdom. However, numerous genes in these pathways are evolutionarily conserved. Modifying these genes (Table 1) has the potential to alter the colors of fish. Presented below are representative examples demonstrating the application of gene editing in changing colors in aquaculture species.

In Atlantic salmon, two genes involved in pigmentation, tyrosinase (*tyr*/TYR) and solute carrier family 45 member 2 (*slc45a2*), were targeted using CRISPR/Cas9 technique (Edvardsen, et al., 2014). At the 17-somite stage, a substantial percentage of embryos (40% for *slc45a2* and 22% for *tyr*) showed significant mutation induction in the targeted genes. Mutated embryos displayed a variety of pigmentation phenotypes, ranging from complete absence to partial loss to normal pigmentation. It was demonstrated that CRISPR/Cas9 could induce double-allelic knockout mutations in the F_0 generation, offering a valuable tool for functional studies. However, the level of mosaicism (genetic variability within an organism) needs to be considered when using F_0 fish for functional studies.

A study focused on investigating the role of the TYR enzyme in melanin synthesis and evaluating the effectiveness of CRISPR/Cas9 for genome modification in red crucian carp (RCC), white crucian carp

(WCC), and their hybrid progeny (Liu, et al., 2019). Utilizing CRISPR/Cas9 technology, the *tyr* gene was selectively targeted and modified in white crucian carp (WCC) and its hybrid offspring with red crucian carp (RCC). This targeted genetic modification led to a decrease in the TYR protein level in the mutant WCC. Both the mutant WCC and mutant hybrid progeny displayed differing extents of melanin reduction when compared to their wild-type counterparts. The degree of reduction ranged from 60% to 90%, indicating different mutation efficiencies in different individuals.

In medaka, the *tyr* gene was also modified to generate a stable albino strain. The mutant medaka exhibited a significant reduction in pigment cells, leading to the generation of albino individuals (Fang, et al., 2018). In the large-scale loach *Paramisgurnus dabryanus*, an economically important aquaculture fish, the *tyr* gene was successfully targeted for editing using the CRISPR/Cas9 system. The resulting mutants exhibited an albino phenotype, enhancing the ornamental and commercial value of the large-scale loach (Xu, et al., 2019).

In common carp, two carotenoid transport genes, *scarb1* (scavenger receptor class B member 1) and *scarb1-like*, were found to be significantly differentially expressed between red and white common carp (Du, et al., 2021). These genes are involved in carotenoid metabolism and transport. Disruption of *scarb1* and *scarb1*-like genes resulted in the fading of regional red skin color (Du, et al., 2021). In contrast, mutations in the *gch1* gene, which is involved in pteridine pigment synthesis, did not affect skin color. When both *scarb1* genes and *gch1* were disrupted together, it led to color variations similar to *scarb1* mutations alone. In the Oujiang color common carp, disruption of agouti signalling protein (*asip*) genes using CRISPR/Cas9 in red individuals with big black patches (RB) resulted in the disappearance of black patches and dispersion of melanophores along the dorsal skin in F_0 mosaic RB individuals (Chen, et al., 2019). These findings suggest *asip* genes play a role in regulating melanin aggregation and distribution during the formation of the black patches. In this species, CRISPR/Cas9 techniques were used to generate melanocortin receptor-1 (*mc1r*) knockout mutants. The loss-of-function (*mc1r*) fish displayed abnormal melanophore production, impaired growth, and alterations in melanogenesis pathways in white and black (WB) color carps (Mandal, et al., 2020).

In tilapia species, many genes involved in pigmentation have been knocked out using CRISPR/Cas9 system to generate preferred colors (Liu, et al., 2022a; Liu et al., 2022b; Sun et al., 2024). Golden Nile tilapia were created by knockouts of premelanosome (*pmela* and *pmelb*), which regulates relative pigment cell abundance, using CRISPR (Wang, Xu, et al., 2022). A study found that the disruption of hermansky-pudlak syndrome 4 (*hps4*) using CRISPR/Cas9 had a profound impact on pigmentation in Nile tilapia, leading to a significant decrease, and in some cases, absence of melanophores and melanin at the adult stage (Wang, Kocher, et al., 2022). This study highlights the fundamental role that *hps4* plays in the pigment development within lysosome-related organelles, melanophore survival, and melanin biosynthesis. Knockout of microphthalmia-associated transcription factor (*mitf*) in Nile tilapia results in a red and yellow tilapia with a reduced number of pigmented melanophores (Wang, Kocher, et al., 2023). The *pmel-17* gene was identified as the gene determining the golden color in Mozambique tilapia (Liu, et al., 2022a; Liu et al., 2022b). Knockout of the *pmel-17* gene in wild type fish resulted in a change of their coloration to golden (Liu, et al., 2022a). The mutants also showed slower growth and faster swimming speed compared to the wild type (Wang, Sun, et al., 2023), suggesting that knockout of *pmel-17* not only affected pigmentation but also other traits. This discovery suggests that when using gene editing to enhance a specific trait, it is prudent to assess other essential traits to prevent potential adverse consequences.

In goldfish (*Carassius auratus*), researchers in China identified the specific genes responsible for two distinct phenotypic variations in goldfish (Yu, et al., 2022). It was determined that the low-density lipoprotein receptor-related protein 2B (*lrp2aB*) gene is the causal gene for the dragon-eye variation, and both homeologs of oculocutaneous albinism type II (*oca2*) are responsible for the albino phenotype. Through gene editing of the identified candidate genes, the dragon-eye and albino phenotypes were successfully recreated in goldfish. The researchers were able to create four stable homozygous strains of goldfish with desirable traits. This suggests the potential for more appealing goldfish varieties for commercial and aesthetic purposes.

The β-carotene 9′, 10′-oxygenase (*Ecbco2*) gene, a crucial enzyme in β-carotene metabolism, was discovered and studied in the prawn species *Exopalaemon carinicauda* (Sun, Liu, et al., 2020). Using CRISPR/Cas9 technology, the *Ecbco2* gene in prawns was successfully deleted by co-microinjecting Cas9 mRNA and ecbco2 sgRNA into one-cell stage embryos (Sun, Liu, et al., 2020). The removal of the *Ecbco2* gene induced a color change in the hepatopancreas of prawns, underscoring the significance of this gene in pigmentation. Prawns with an *Ecbco2* knockout demonstrated an increased survival rate compared to their wild-type counterparts when exposed to pathogenic bacteria such as *Vibrio parahaemolyticus* or *Aeromonas hydrophila*. These findings show the potential utility of *Ecbco2* as a candidate gene in the molecular marker-assisted breeding of prawns.

CRISPR/Cas9 has enabled significant advancements in modifying pigmentation in aquaculture species such as creating fish with altered pigment patterns for aesthetic purposes, enhancing camouflage, and reducing visibility to predators. In addition, targeted gene editing has led to the development of albinism-free strains, improving resistance to UV radiation and certain diseases. These genetic modifications hold promise for both commercial aquaculture and conservation, as manipulating pigmentation can impact fish health, survivability, and market appeal. Utilizing conserved genes within pigmentation-related pathways appears feasible for pigment modification through gene editing However, it is essential to evaluate whether such editing will affect other economically important traits. In ornamental fish, the application of gene editing facilitates the precise selection of preferred colors, which has significant importance in the aquarium industry (Yu, et al., 2022; Zhang et al., 2023). It enables the creation of vibrant and unique fish variants, increasing their aesthetic appeal to hobbyists and collectors. This innovation not only meets market demand for visually appealing fish but also reduces the capture of wild species, aiding in conservation efforts. Furthermore, gene editing can enhance the overall health of ornamental fish, making them more resilient to diseases. By promoting sustainable and ethical practices, gene-edited color variations in ornamental fish contribute to the preservation of biodiversity, the well-being of captive fish populations, and the economic sustainability of the aquarium trade.

4.7. Edited genes for other traits

Gene editing has been used to improve other traits, which are important for the aquaculture industry. This section offers an overview of key findings from representative studies in this area.

The modification of *igfbp-2b* genes in rainbow trout led to an 83% reduction in serum *igfbp-2b* levels among the mutants. Rainbow trout that were gene edited to lower their serum insulin-like growth factor binding protein (IGFBP)-2b levels displayed growth performance comparable to that of fish without disruptions in the *igfbp-2b* gene (Cleveland, et al., 2020). A recent study conducted in China showed that the deletion of the *t1r1* gene significantly increased the acceptance of plant proteins in zebrafish (Cai, et al., 2021). Chitinases, part of the O-glycoside hydrolase superfamily, efficiently break down chitin. Specific enzyme combinations can be used to yield desired oligomer lengths. Chitooligosaccharides require more endochitinase and less exochitinase/N-acetyl-glucosaminidase, while N-Acetylglucosamine (GlcNAc) production requires more exochitinase/N-acetyl-glucosaminidase. Chitinases also find use in single-cell protein, protoplast isolation, pest control, and plant fungal disease resistance through

genetic engineering (Dahiya, et al., 2006). In 2016, Chinese researchers achieved the first genome editing of a decapod, the ridgetail white prawn *Exopalaemon carinicauda* (Gui, et al., 2016). Their objective was to elucidate the characteristics of the chitinase compound EcChi4 by disabling the production of its gene, *ecchi4*. Embryos at the one-cell stage, obtained from recently spawned females, were gathered, subjected to microinjection, and subsequently incubated for a period of 15 days. The genome of the shrimp that hatched was analysed, revealing a mutation rate of 7.3%. Mendelian inheritance was observed when breeding heterozygous mutant shrimp. The *ecchi4* mutation was heritable without affecting survival or growth.

Ammonia nitrogen is highly toxic to fish, and studies often focus on its damaging effects, but few explore ways to enhance ammonia tolerance in fish. A study was conducted to investigate the effects of ammonia nitrogen exposure on apoptosis, endoplasmic reticulum (ER) stress, and immune cells in loach (*Misgurnus anguillicaudatus*). A chop-depleted loach model was created using CRISPR/Cas9(Lv, et al., 2023). Ammonia nitrogen stress caused down-regulation of apoptosis-related genes in the gills of *chop* $^{\pm}$ loach. The *chop* $^{\pm}$ loach also had higher levels of immunity-related cells and increased survival under NH_4Cl exposure, suggesting that inhibiting the function of chop strengthened the innate immune barrier (Lv, et al., 2023). These findings pave the way for developing germplasm with high ammonia nitrogen tolerance in aquaculture.

In aquaculture species, other than economic traits, traits such as robustness (Agha, et al., 2018) may also play an important role in fish adaptation to changing environment. One example of gene editing for environmental adaptation in aquaculture species involves modifying fish to thrive in brackish or saline water environments. Tilapia is a commonly farmed species, and researchers have used gene editing to enhance its ability to tolerate higher salinity levels, which expands the range of aquaculture sites and reduces freshwater usage.

5. Edited genes in cell lines to analyse their functions

Some genes linked to crucial traits in aquaculture species have been studied by editing them in cell lines (Table 2). In this section, we outline the main discoveries and challenges when examining gene functions through editing in cell lines.

In a study, CRISPR/Cas9 technology was used to knock out the grass carp Junctional Adhesion Molecule-A (*gcjam-a*) and assess its resistance against GCRV genotypes in vitro (Ma, et al., 2018). The application of CRISPR/Cas9 successfully disrupted *gcjam-a*, leading to a reduction in GCRV infection in grass carp kidney cells (CIK) for two genotypes. This was evidenced by decreased cytopathic effects (CPE) and reduced GCRV production. Furthermore, the introduction of the expression of ectopic *gcjam-a* into non-permissive Chinese giant salamander muscle cells enabled infection by both GCRV-JX0901 and Hubei grass carp disease reovirus (HGDRV) strains. These findings underscore the essential role of *gcjam-a* in GCRV infection, offering potential implications for viral control in aquaculture(Ma, et al., 2018). Analysis of gene expression showed that NEDD-8 activating enzyme 1 (*nae1*) is a likely functional candidate associated with the identified quantitative trait loci (QTL). When *nae1* was knocked out using CRISPR/Cas9 or chemically inhibited in cell lines, a notable decrease in in infectious pancreatic necrosis virus (IPNV) replication was observed (Pavelin, et al., 2021). These findings strongly imply that *nae1* is the key gene responsible for the major QTL influencing resistance to IPNV in salmon.

A subset of specialized cytokines crucial for virus immunity includes Interferon (IFN) (Dehler, et al., 2019). Upon viral infection, the production of type I IFN triggers changes in the transcriptome of responsive cells, activating a set of IFN-stimulated genes (ISGs) with regulatory or antiviral functions. This leads to the establishment of a cellular antiviral state. In the GS2 cell line of Chinook salmon (*Oncorhynchus tshawytscha*), the *stat2* gene was intentionally disabled using CRISPR/Cas9 genome editing (Dehler, et al., 2019). Unexpectedly, the induction of ISGs through stimulation with recombinant type I IFN is abolished in this cell line, as evidenced by a comparative RNA-seq analysis of the transcriptome between GS2 and its parental counterpart. Despite the complete absence of ISG induction, the GS2 cell line demonstrated remarkable resistance to viral infections. This suggests that viral infections may trigger alternative STAT2-independent pathways, highlighting the robustness and redundancy of innate antiviral defences in fish. *Maf1*, a global RNA polymerase III-dependent transcription suppressor found in organisms ranging from yeast to humans, has implications in mammalian immune responses (Kim, et al., 2021). However, its role in fish remains unknown. In the olive flounder (*Paralichthys olivaceus*), *po-maf1* was knocked out in HINAE cells using the CRISPR/Cas9 system. Knockout of *pomaf1* increased viral hemorrhagic septicemia virus (VHSV) glycoprotein (G) mRNA levels upon infection.

The upregulation of *mx1* and *isg15* genes in *Epithelioma papulosum cyprini* (EPC) cells in response to type I interferon is known. Their antiviral roles against *Hemorrhagic septicemia* virus (VHSV) were investigated using CRISPR/Cas9 knockout (Kim & Kim, 2019b). *Mx1* knockout showed no impact on VHSV-induced cytopathic effects, suggesting limited functionality against cytoplasmic RNA viruses. In contrast, *isg15* knockout cells exhibited compromised antiviral activity, emphasizing *isg15*'s crucial role in type I interferon-mediated responses. VHSV replication was more efficient in ISG15 knockout cells compared to *mx1* knockout and control cells (Kim & Kim, 2019b). In addition, CRISPR/Cas9 was used to knockout *hif-1α* in EPC cells, in order to investigate the role of hypoxia-inducible factor-1 (*hif-1*) in fish cell apoptosis (Kim & Kim, 2018a). The single cell clone with a heterozygous insertion/deletion mutation was selected. Luminescence assays with a hypoxia reporting vector confirmed *hif-1α* knockout, as the cells showed no response to cobalt chloride exposure. *Hif-1α* knockout significantly inhibited apoptosis induced by camptothecin and viral hemorrhagic septicemia virus (VHSV) infection, and VHSV replication was slowed in *hif-1α* knockout EPC cells (Kim & Kim, 2018a). These findings suggest that

Table 2
A list of edited genes for functional analysis in cell lines in aquaculture species.

Economic traits	Disease [a]	Genes	Species	References
Disease resistance	Reovirus	*jam-a*	*Ctenopharyngodon idella*	Ma, et al. (2018)
	pancreatic necrosis virus	*nae1*	*Salmo salar*	Pavelin, et al. (2021)
	SPDV, VHSV and EHNV.	*stat2*	*Oncorhynchus tshawytscha*	Dehler, et al. (2019)
		maf1	*Paralichthys olivaceus*	Kim, et al. (2021)
	VHSV	*mx1* or *isg15*	*Epithelioma papulosum cyprini*	(Kim & Kim, 2019b),
	VHSV	*hif-1α*	*Epithelioma papulosum cyprini*	(Kim & Kim, 2018a),
	VHSV	*traf6*	*Epithelioma papulosum cyprini*	(Abdellaoui, et al., 2023),
	VHSV	*irf9*	*Epithelioma papulosum cyprini*	(Kim, et al., 2018b),
	VHSV	*irf-3 and irf-5*	*Epithelioma papulosum cyprini*	(Kwak et al., 2023)
	IPNV and SAV3	*mavs*	*Salmo salar*	(Ma, et al., 2018; Van Der Wal et al., 2023)
	SGIV	*lcalmnb2*	*Lates calcarifer*	Yu, et al. (2024)
	NNV	*pIgR*	*Lates calcarifer*	Tay, et al. (2023)
	SGIV	*snx2*	*Lates calcarifer*	Yu, et al. (2021)

[a] SPDV: Pancreas disease virus, VHSV: Viral hemorrhagic septicemia virus, EHNV: epizootic hematopoietic necrosis virus, IPVN: infectious pancreatic necrosis virus, SAV3: alphavirus subtype 3, SGIV: Singapore grouper iridovirus, and NNV: nervous necrosis virus.

HIF-1α acts as a pro-apoptotic factor in EPC cells that are triggered by DNA damage and viral infection during apoptosis. Furthermore, the introduction of genetic modifications in EPC cells resulted in the knockout of several key genes, such as *traf6* (Abdellaoui, et al., 2023), *irf9* (Kim, et al., 2018b), *irf3* and *irf5* (Kwak and Kim, 2023). This targeted genetic manipulation not only contributes to the improvement of the cellular immune system but also enhances the cells' resilience against viral infections.

The interferon (IFN) responses play a crucial role in resolving viral infections and are frequently targeted by various viruses. MAVS in IFN signalling was deliberately disrupted within the salmonid cell line CHSE-214 using CRISPR/Cas9. This resulted in the successful generation of knockout clones for MAVS, as well as for the transcription factors *irf3, irf7-1*, and a double knockout for *irf7-1/3* (Van Der Wal, et al., 2023). *Mavs* and *irf3* knockouts in CHSE-214 cells inhibited IFN and IFN-stimulated gene induction after poly I:C stimulation, while *irf7-1* knockout had no clear effect. Salmon alphavirus 3 (SAV3) infection in control and *irf7-1* KO cells yielded similar titers and no cytopathic effect, while *irf3* and MAVS KOs showed severe cytopathic effects and increased titers. IPNV yields were reduced in *irf3* and *mavs* KOs, suggesting a dependence on interactions between viral proteins and pattern recognition receptor signalling components during viral replication (Van Der Wal, et al., 2023).

These examples highlight the diversity of genes targeted for gene editing in aquaculture species, depending on the specific goals of the breeding program and the species in question. The genetic basis of economic traits in aquaculture species is a complex and evolving field of research. Specific genes associated with economic traits can vary among different species and strains.

6. Limitations of gene editing in aquaculture

The applications of CRISPR/Cas in fish aquaculture faces limitations due to limited genomic resource. While the genomes of important aquatic species have been sequenced, there is still a lack of genomic sequences for many aquaculture species. Although over 70 fish species have had their genomes sequenced (Abdelrahman, et al., 2017; Yue & Wang, 2017), this is a small fraction compared to the estimated total number of aquaculture species, which exceeds 600 (FAO, 2022). Furthermore, the sequenced genomes are still not well annotated for non-model species, which calls for further refinement in the application of CRISPR/Cas in aquaculture. The lack of information regarding trait-causative genes is another limitation. Unlike genetic studies in humans, plants and livestock, research on trait-causative genes in aquatic organisms is still limited (Abdelrahman, et al., 2017; Yue & Wang, 2017). The identification of target genes through processes like QTL mapping or GWAS is time-consuming (Yang, Yu, Wang, et al., 2022; Yue, 2014), and the precise identification of causative genes for polygenic traits remains challenging (Yang, et al., 2023b). In addition, fish undergo genome duplication, which poses a problem in genetic studies (Meyer & Van de Peer, 2005). Teleost, a class of fish, has undergone a teleost-specific whole genome duplication (TS-WGD), with salmon, in particular, experiencing a salmon-specific fourth round of genome duplication (Ss4R) (Lien, et al., 2016). This duplication hampers the efficiency of genome editing techniques in finfish. Comparing genes with different copy numbers in the genome could shed light on the implications of duplication in gene editing efficiency.

Limitations in the use of CRISPR/Cas in fish aquaculture arise from challenges such as the low success rate of microinjection in oviparous fish due to the egg membrane. In addition, there is a lack of an established gene editing platform for ovoviviparous fishes, adding to the limitations in their genetic manipulation. Furthermore, the long generation interval in many aquatic species makes the generation of mutated homozygous individuals time-consuming during the gene editing process. Surrogacy (Yoshizaki & Yazawa, 2019) presents a potential solution to address this limitation by combining gene editing with offspring production through surrogate parents. The preference for utilizing sterile organisms in commercial applications arises from the need to protect intellectual property and prevent the unintentional release of gene-edited individuals into the wild population. However, sustaining heterozygous individuals for population maintenance requires increased efforts. In a recent study, Güralp and collaborators developed a technique capable of rescuing germ cells in *dnd* crispant-embryos of Atlantic salmon. This facilitates the production of sterile offspring—devoid of germ cells—via genetically sterile broodstock (Güralp, et al., 2020).

In summary, while gene editing holds great promise for improving the sustainability and productivity of aquaculture, it also presents complex challenges related to regulation, ethics, unintended consequences, and public acceptance. Successful and responsible implementation of gene editing in aquaculture will require a careful balance of scientific innovation, regulatory oversight, and public engagement to address these challenges and maximize the benefits.

7. Finding genes for gene editing

The search for causative genes for economically important traits centres on three major techniques, such as the candidate gene approach, QTL mapping and GWAS (Houston, et al., 2020; Yue, 2014) (Fig. 3).

The candidate gene approach is a targeted method used to identify specific genes responsible for important traits in agronomic species (Tabor, et al., 2002). It involves selecting genes that are believed to play a role in the trait of interest based on prior knowledge, such as their function in related species or their involvement in relevant biochemical pathways (Xu, et al., 2006). By focusing on specific candidate genes, this approach can streamline the search for causative genes, making it a valuable tool in genetic improvement and breeding programs (De-Santis et al., 2007). The initial and crucial step in conducting candidate gene studies involves selecting an appropriate candidate gene that is likely to have a significant role in the process or disease being investigated. For instance, when examining genes associated with growth traits, the myostatin gene is a logical choice (Aiello, et al., 2018; McPherron and Lee, 1997; Ou et al., 2023). This is supported by findings in livestock where polymorphisms in this gene have been linked to growth traits. It is widely recognized that myostatin acts as a negative regulator for growth.

After selecting a candidate gene, the subsequent decision involves determining the most suitable polymorphism for evaluation in an association study (Yue, et al., 2001). In this context, researchers must identify extant gene variants and ascertain which of these variants lead to modifications in proteins that could potentially impact the trait of interest. Polymorphisms that cause changes in proteins that contribute to the development of a distinct phenotype hold significance in candidate gene studies (De-Santis et al., 2007). Nevertheless, it is essential to note that most mutations do not lead to alterations in amino acids or affect the functionality of the resultant protein (Wang, et al., 2021; Wang, Sun, et al., 2022). Moreover, numerous mutations occur in noncoding DNA regions, resulting in alterations that are not associated with protein variants linked to a modified phenotype (Wang, et al., 2021; Wang, Sun, et al., 2022). However, mutations occurring in noncoding regions can still provide value in candidate gene studies. Changes in regulatory regions, such as promoters or intron splice sites, have the potential to modify gene activity and, consequently, influence the phenotype governed by that gene (Nordborg & Tavaré, 2002; Tabor et al., 2002). Identifying genetic variants is a labour-intensive process that typically involves sequencing. This method requires determining the sequence of DNA building blocks (i.e., nucleotides) for the entire gene in both affected and unaffected individuals to identify consistent differences. (Tabor, et al., 2002). For a variant to be applicable in candidate gene

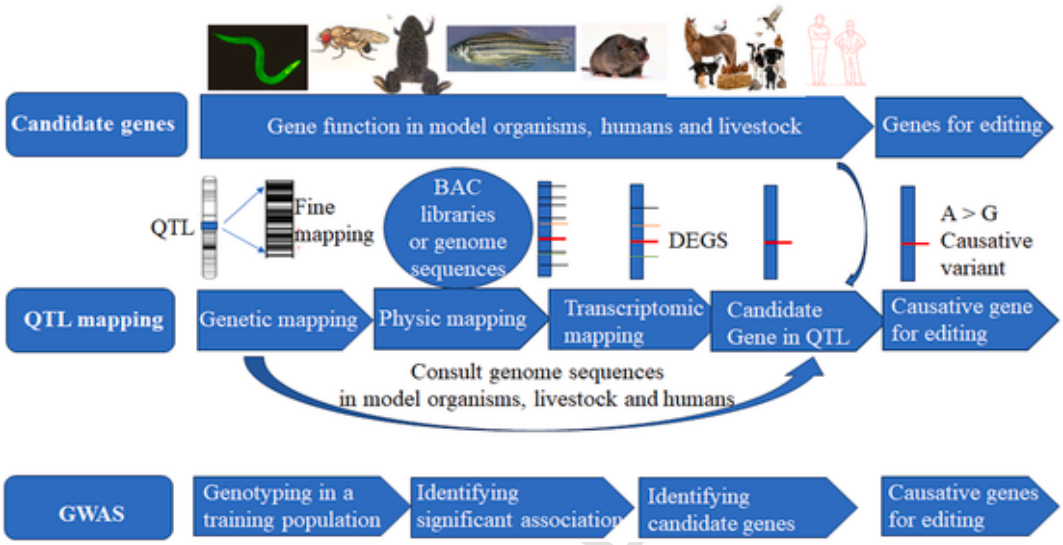

Fig. 3. A roadmap of identifying gene responsible for important traits for editing through three distinct approaches.
The upper section outlines the candidate genes approach, emphasizing the selection based on their functions in model organisms, humans, and livestock. The middle section delineates the QTL mapping and positional cloning approach, involving gene identification through QTL mapping in pedigrees, along with positional cloning utilizing transcriptomes, BAC libraries, and whole-genome sequencing. The lower section details the genome-wide association studies (GWAS) approach, where genes are identified through GWAS encompassing many individuals (>1000 fish) and numerous DNA markers (>10,000 SNPs) covering the entire genome of the species under investigation.

studies, it must manifest with sufficient frequency to facilitate the differentiation between individuals with and without the investigated trait. It is worth noting that not all genes possess readily identifiable common functional variants suitable for association studies. In numerous instances, researchers may only pinpoint SNPs with no known functional significance (Fu, et al., 2013; Fu et al., 2014; Xia et al., 2013; Yang et al., 2020; Yu et al., 2021). However, SNPs can remain valuable tools in refining a linkage region and may demonstrate a statistically significant association with a gene associated with disease susceptibility if they are positioned within or near that gene, owing to linkage disequilibrium. The challenge lies in candidate genes, as they may not be universally conserved across different fish species. Gene functions have diversified, especially in fish, due to multiple rounds of genome duplication during evolution (Venkatesh, 2003). Therefore, cautions must be taken when using candidate genes for knockout in new species.

QTL mapping is a powerful tool used in genetic studies to identify the genomic regions associated with particular traits of interest, such as economic traits in aquaculture species (Yue, 2014). In the following, we outline the method used to identify important genes for economic traits using QTL mapping. Data on the economic traits of interest such as growth rate, disease resistance, feed efficiency and yield were gathered by measuring these traits on multiple individuals from the selected aquaculture species. A set of DNA markers, such as microsatellites, SNPs, or other genetic markers, with known positions on a genetic or physical map, and are evenly distributed across the genome were selected. DNA was extracted from the study population and genotyped with the selected markers using PCR, DNA sequencing, or genotyping arrays. Statistical analysis was performed using software packages specifically designed for QTL mapping such as QTL Cartographer (Basten, et al., 2002), R/QTL (Broman & Sen, 2009), or MapQTL (Van Ooien, et al., 2000), linking individual genotypes to phenotypic data, to identify genome regions (QTL) that are significantly associated with the economic traits. The identified QTL were validated by repeating the study on independent populations or through experimental crosses (Wang, et al., 2019) to ensure robust and reproducible results. To narrow down the identified regions and identify specific genes responsible for the observed traits, additional genotyping with higher density markers, re-sequencing the regions of interest, or analysing gene expression patterns was conducted. Finally, the candidate genes can be functionally characterized by studying the gene expression patterns (Li, et al., 2004), gene knockdowns or overexpression experiments (Yang, Yu, Wang, et al., 2022; Yang et al., 2023a), or functional annotations using available databases. QTL mapping can assist in uncovering the genetic basis of economically important traits in aquaculture species, aiding in the breeding or biotechnological improvement of these species to enhance productivity and profitability (Yue & Wang, 2017). For example, the *gab3* gene, which plays an important role in resistance against NNV in Asian seabass, was identified by QTL mapping, fine mapping and functional analysis in a cell lines. Knockout of this gene in fish led to reduced mortality after NNV challenge (Yang, et al., 2020; Yang, Yu, Wang, et al., 2022). In tilapia, the *pmel-17* gene determining the golden colour was identified using the same strategies. Knockout of this gene in wild type Nile tilapia resulted in the change of colors from blackish to golden (Liu, et al., 2022a; Liu et al., 2022b; Wang, Xu, et al., 2022).

GWAS is a genetic investigation strategy used to identify associations between genetic variations, usually single nucleotide polymorphisms (SNPs), and specific traits or diseases within a population (Tam, et al., 2019). GWAS functions as a powerful tool for uncovering the genetic basis of complex traits and diseases, with applications in various fields, including genetics, genomics, medicine, and agriculture. An overview of GWAS methodology involves initiating the study with a sample population comprising of individuals with diverse genetic backgrounds, usually unrelated and representative of the broader population under investigation. For instance, a population designed for the study of a specific disease or trait includes both affected and unaffected individuals. GWAS workflow entails phenotyping, genotyping, statical analysis, correction for multiple testing, and visualization (Tam, et al., 2019; Wang et al., 2017). Following the identification of significant associations, the genomic regions around the associated SNPs are explored to identify candidate genes that might be responsible for the observed trait or disease associations (Yu, Wang, et al., 2019). Candidate genes are further investigated to understand their biological role in the observed trait or disease. This may involve experiments to confirm the functional relevance of the genes. To validate the initial findings, repli-

cation studies in independent populations are often performed to confirm the reliability of the associations. It is noteworthy that GWAS is constitutes the initial step in the gene discovery and editing process. Comprehensive functional validation is crucial to confirm the roles of candidate genes (Yang, et al., 2023b), Yang et al., 2023bnd field trials are necessary to evaluate the real-world performance of edited plants. Collaboration with experts in genetics, genomics, and gene editing is also highly recommended for the success of the project.

In conclusion, there are a variety of methods such as the candidate gene approach, QTL mapping, linkage mapping, and GWAS, that have proven to be the effective in identifying genes responsible for crucial traits in aquaculture species. The candidate gene approach is valuable for swiftly establishing links between genetic variants and specific traits, especially when dealing with genes of moderate impact. This method has distinct advantages over traditional approaches like linkage mapping and positional cloning, as well as GWAS. With the continuous discovery of SNPs distributed across the genome, researchers can also apply the candidate gene approach on a comprehensive, genome-wide scale, further enhancing its utility in advancing aquaculture genetics.

8. Integrating gene editing in aquaculture breeding and production

Integrating gene editing in aquaculture breeding and production involves leveraging cutting-edge genetic tools to enhance desirable traits in farmed fish species. This process, often employing CRISPR-Cas9 technology, enables precise modification of specific genes linked to traits like disease resistance, growth rate, and environmental adaptability.

An exemplary instance of this integration can be observed in the development of bone-free mutants within amphitriploid gibel carp and blunt snout bream. In amphitriploid gibel carp, this milestone was attained by editing two duplicated runx2b homeologs (Gan, et al., 2023). Similarly, the creation of bone-free Blunt snout bream was accomplished by mutating the runx2b gene (Dong, et al., 2023). These genetically modified fish are now actively involved in breeding programs aimed at enhancing various other traits in both species. Another successful instance of this integration is evident in the cultivation of rapid-growth red sea bream and tiger puffer (*Takifugu rubripes*) for commercial production in Japan. (Kishimoto, et al., 2019; NEWs, 2022). Another successful instance of this integration is evident in the cultivation of rapid-growth red sea bream and tiger puffer (Takifugu rubripes) for commercial production in Japan (Kishimoto, et al., 2019). In Japan, approval for sale has been granted to two fish species that have undergone mstn editing: the tiger puffer fish and the red sea bream (NEWs, 2022).

However, the widespread adoption of gene editing in aquaculture necessitates addressing regulatory, ethical, and public acceptance considerations. Clear guidelines and frameworks must be established to ensure the responsible and transparent application of gene editing technologies in aquaculture, prioritizing both environmental sustainability and animal welfare. Overall, integrating gene editing holds immense promise for advancing the productivity, resilience, and sustainability of aquaculture systems, while also meeting the growing global demand for seafood.

9. Future perspectives and conclusion

The transformative potential of gene editing in aquaculture is undeniable with certain key areas holding immense promise for the future. These areas include: 1. Disease resistance: gene editing provides an opportunity to increase the disease resistance of aquaculture species. By targeting specific genes associated with disease susceptibility, strains that are more resilient to common pathogens can be developed, thereby reducing the economic losses caused by disease outbreaks. 2. Improved growth and feed conversion: Gene editing can be used to enhance the growth characteristics and feed efficiency of aquaculture species. By modifying genes responsible for growth regulation or metabolic processes, strains that grow faster and convert feed more efficiently can be developed, leading to increased productivity, and reduced environmental impact. 3. Environmental adaptation: Aquaculture species often need to adapt to various environmental conditions. Gene editing can enable the development of strains that are better suited to specific environments, such as tolerance to high-temperature, salinity tolerance, or resistance to hypoxic conditions, expanding the geographical range for aquaculture production. 4. Enhanced nutritional quality: Gene editing can be used to improve the nutritional quality of aquaculture species. By modifying genes involved in lipid synthesis, amino acid profiles, or nutrient utilization, researchers can develop fish with improved nutritional composition, providing healthier food options. 5. Conservation efforts: Gene editing has the potential to contribute to conservation efforts for endangered or threatened aquaculture. species. It may increase reproductive success, leading to increased genetic diversity and the reintroduction of desirable traits, thereby supporting the recovery of populations in the wild.

Despite its potential, challenges remain. Many important traits are complex and are influenced by multiple genes and environmental factors. Identifying and functionally analysing genes for key traits is crucial for targeted editing. In addition, improved methodologies are needed to increase editing efficiency, survival rates, and reduce off-target effects. Ethical, safety, and regulatory considerations must also be addressed. Balancing economic viability with environmental sustainability requires a multidisciplinary approach and a commitment to responsible innovation. Gene editing is a powerful tool, and responsible use requires acknowledging ethical and ecological responsibilities. By balancing scientific advancements with ethical considerations and regulatory oversight, we can ensure that gene editing leads to a more efficient and sustainable future for aquaculture.

Data availability statements

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

CRediT authorship contribution statement

Zituo Yang: Writing – review & editing, Writing – original draft, Visualization, Data curation, Conceptualization. **Guihong Fu:** Writing – review & editing, Writing – original draft, Conceptualization. **May Lee:** Writing – review & editing, Writing – original draft. **Shadame Yeo:** Writing – review & editing, Writing – original draft. **Gen Hua Yue:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

All authors declare no conflicts of interest.

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