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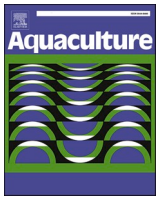

Review

Harnessing CRISPR/Cas9 system to improve economic traits in aquaculture species

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ABSTRACT

Clustered regularly interspaced short palindromic repeats associated with Cas9 (CRISPR/Cas9) system is a powerful and efficient tool for molecular modification in the genome using programable RNAs. It holds a wide application in plant and animal biotechnology for enhancing multiple traits of interest. Here, we attempted to gather and review the recent progress and provide an overview of the application benefits of this technology in aquatic animal genomes. In this respect, several studies were performed on different species (marine and freshwater) investigating the growth bioprocess trait, wherein, the majority of them focused on somatic axis suppressors such as *mstn* (myostatin) knock-outs and their effects on the differentiation and proliferation of somatic cells. Regarding the reproduction feature, the retrieved studies provide insights into germ cells, sex reversal, and fertility mechanisms with more focus on zebrafish and tilapia compared to other species. In turn, a generation of *de novo* strains more resistant to viral infections has been successfully attended to proving the practical ability of this technology in enhancing the immunity of certain species such as grass carp. Furthermore, multiple experiments were performed to explore the pigmentation particularity and understand the melanin biosynthesis pathway via *tyr* (tyrosinase) gene knock-out in different freshwater species. To investigate the metabolism processes, researchers attempted to grasp the molecular mechanisms underlying polyunsaturated fatty acids biosynthesis and lipid metabolism in Atlantic salmon and channel catfish. In another aspect, earlier studies were undertaken in cyprinids to produce intramuscular bone-free populations as an important trait of interest. Finally, we examined the possible challenges and perspectives for the future use of this technology in the research field. Overall, the application of CRISPR/Cas9 system is an effective approach to manipulating target genes and enhancing economic traits in different aquatic animal species, thereby strengthening genetic breeding programs.

1. Introduction

Nowadays, fisheries resources are in a plateau or decline situation, whereas the world population and global demand for this nutritional source are on an increasing trend. To overcome this challenge, aquaculture emerges as an alternative sub-sector and it can be seen as highly developed in some Asian countries and still in its infancy stage in some other geographical areas. Thus, the harnessing of applied sciences to boost this sub-sector from traditional practices and facilities to modern ones in order to increase production and enhance its quality is encouraging. In tandem, genetic breeding, aquatic animal nutrition and disease, aquatic environment, and aquaculture engineering, all of which are research areas that contributed to the flourishing and burgeoning of this industry. In this regard, the application of genetics, selective breeding, and genome editing in aquaculture provides exciting avenues to accelerate the genetic improvement of aquaculture species (Jin et al., 2021), and may potentially create *de novo* strains, or introduce advantageous alleles (Gratacap et al., 2020; Gratacap et al., 2019). The main applied genetic engineering tools used in aquaculture functional studies are ZFN (zinc finger nuclease), TALEN (transcription activator-like effector nuclease), and CRISPR/Cas9. These techniques have been adopted to study and explore some complex traits such as growth, reproduction, pigmentation, disease resistance, metabolism, and environmental tolerances. Thereof, in aquatic conventional selective breeding, CRISPR/Cas9 system is integrated into breeding programs and considered a potential and promising tool to play subtle functions in the

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enhancement of economic traits (Yang et al., 2022), thereby, enhancing the production either by inducing beneficial alleles into cultured fishes or indirectly by coming up with a better comprehension of the practical basis of traits (Macqueen et al., 2017). Compared to ZFN and TALEN, CRISPR/Cas9 is considered the more convenient, efficient, and cost-effective approach (Tao et al., 2021), and due to its large advantages including targeted mutagenesis, this system has been extensively used since 2013 (Cong et al., 2013). This, in turn, proved its good editing efficiency, higher target specificity, and minor adverse effects in the most studied aquatic animal species. In addition, it represents an up-and-coming tool that is used to produce transgenic fish instead of transferring plasmid DNA into embryos as declared by Xing et al. (2022a). The objective of this paper is to review and summarize the research progress of functional studies that have been performed to improve different traits of interest in aquaculture species via the application of CRISPR/Cas9 system.

2. Mechanism of delivery

Conducting specific changes in the genome of the target species via CRISPR/Cas9 technology may facilitate the identification and characterization of specific operating variants thereby affecting the trait of interest (Gratacap et al., 2019). Hence, to deliver CRISPR/Cas9 in the cell, three common strategies are used, the first method is to apply a plasmid-based CRISPR-Cas9 system encoding Cas9 protein and single-guide RNA (sgRNA) as a vector (Ran et al., 2013). The second is to induce the Cas9 mRNA and sgRNA mixtures into cells (Niu et al., 2014; van Hees et al., 2022). The third form is Cas9/sgRNA ribonucleoprotein (RNPs) complex, which is expected to promptly move in the nucleus and cleave the target sequence (Hiruta et al., 2018; Hiruta et al., 2013). Compared to method one and method two, sgRNA/Cas9 RNP complex was declared to be an efficient and simple approach for mediating gene mutations (Yu et al., 2019). This has been proven in different studies carried out in some aquatic animals such as common carp (*Cyprinus carpio*) and Pacific oyster (*Crassostrea gigas*) (Shahi et al., 2022; Yu et al., 2019). Moreover, to transfect CRISPR/Cas9 physically, microinjection, electroporation, and hydrodynamic injection are the commonly adopted methods and the main principle of these techniques is to ensure the delivery of gene cargo directly to target cells and avoid any possible challenges related to immunogenicity and endocytotic pathways (Rahimi et al., 2020)

3. Application of CRISPR/Cas9 in aquaculture

Six main traits have been targeted via CRISPR/Cas9 system to precisely induce mutagenesis in the genetic code of aquatic animal species as summarized in Table 1. Notably, the application of this technology was reported with a main focus on freshwater species such as model fish (medaka and zebrafish) and tilapia (*Oreochromis niloticus*), however, there were no functional studies that have been reported in crustacean species using this technology up to now.

3.1. Growth and muscle development

The biological process of somatic growth is controlled and influenced by nutritional factors, hormones as well as multiple physiological and molecular mechanisms that ensure cell proliferation and differentiation, thereby the development of muscles and bones. In this regard, emerging evidence suggested the up-regulation of somatotropic genes including *IGF* (Insulin-like Growth Factor) and *GH* (Growth Hormone) may improve the hyperplasia of muscle cells (Gao et al., 2016; Li et al., 2014a; Wang et al., 2001). Additionally, knockouts of certain somatic axis suppressors genes such as *mstn* (myostatin) and *socs1a* could also enhance fish growth (Chisada et al., 2011; Dai et al., 2015; Gao et al., 2016; Li et al., 2012). Accordingly, it's common saying that *mstn* is regularly expressed in skeletal muscle as a negative modulator of

Table 1
Application of CRISPR/Cas9 approach in aquaculture with respect to specific traits, species, mutated genes, and main effects.

Traits	Species	Mutated genes	Main effects	Reference
Growth	Common carp	*mstn, sp7*	- Increase in body weight and length. - Bone anomalies.	(Zhong et al., 2016)
		mstn	- Double muscle phenotype in the produced F0.	(Shahi et al., 2022)
	Medaka	*mstn*	- Significant increase in body weight and length. - Scoliosis	(Yeh et al., 2017)
	Blunt snout bream	*mstn*	- Increase in body weight and muscle development.	(Sun et al., 2020)
	Channel catfish	*mstn*	- Increase in muscle hyperplasia and body weight. - Significant increase in body weight.	(Khalil et al., 2017) (Coogan et al., 2022)
	Red sea bream	*mstn*	- Increase in skeletal muscle mass.	(Kishimoto et al., 2018)
	Olive flounder	*mstn*	- Hyperplasia and body thickness.	(Kim et al., 2019)
	Loach	*mstn*	- Somatic growth with lipid accumulation in loach.	(Tao et al., 2021)
	Pacific oyster	*MELC*	- Effect on of larval muscle myogenesis.	(Li et al., 2021a)
		MELC	- Effect on sarcomeric organization of thin filaments	(Li et al., 2021b)
		smyhc, hsp90α1	- Inhibition of myosin thick filament formation.	(Cai et al., 2018)
	Zebrafish	*pomc, acvr2*	- Muscle fibers development and enhancing growth performance	(Che et al., 2023; Yang et al., 2023)
	Tilapia	*mstn*	- Double-muscle phenotype with increased muscle mass.	(Wu et al., 2023)
		dmrt1	- Total sex reversal and inhibition of estrogen production.	(Jiang et al., 2016)
		dmrt6	- Reduction in 11-KT level and spermatocytes.	(Zhang et al., 2014)
		sf-1	- Sex reversal.	(Xie et al., 2016)
		stra8	- Regulating sex determination.	(Feng et al., 2015)
Reproduction	Tilapia	*amhy*	- Sex reversal.	(Li et al., 2015)
		wt1a, wt1b	- Urogenital system development.	(Jiang et al., 2017)
		nanos2, nanos3	- Germ cells loss.	(Li et al., 2014b)
		dmrt1, fox1, foxl2 and cyp19a1a	- Regulation of sexual plasticity of germ cells.	(Dai et al., 2021)
		gsdf	- Sex reversal.	(Jiang et al., 2016)

(*continued on next page*)

Table 1 (*continued*)

Traits	Species	Mutated genes	Main effects	Reference
		foxh1	- Oogenesis arrest and female sterility.	(Tao et al., 2020)
		eEF1A1b	- Arrest and a reduction in male fertility.	(Chen et al., 2017)
		Esr2a, Esr2b	- Sex reversal.	(Lu et al., 2017)
	Zebrafish	*cyp11c1*	- Insufficient spermatogenesis and a delay in ovary-to-testis transition.	(Zhang et al., 2020)
	Common carp	*cyp17a1*	- Sex reversal.	(Zhai et al., 2022)
	Yellow catfish	*pfpdz1*	- Male sex differentiation and maintenance.	(Dan et al., 2018)
	Medaka	*dnd*	- Germ cell loss.	(Zhu et al., 2018)
	Rainbow trout	*dnd*	- Germ cell loss.	(Fujihara et al., 2022)
		JAM-A	- Resistance against GCRV.	(Ma et al., 2018)
	Grass carp	*itgb1*	- Limitation of viral entry into the cells.	(Chen et al., 2018)
	Olive flounder	*Maf1*	- Immune response.	(Kim et al., 2021)
Disease resistance	Chinook salmon	*stat2*	- Effect on IFN-I signaling.	(Dehler et al., 2019)
	Zebrafish	*prmt7*	- Resistance to viral infection.	(Zhu et al., 2020)
	Channel catfish	*Cath*	- High integration rate (disease resistance).	(Simora et al., 2020)
	Chinook salmon	*Stat2*	- Immune response.	(Dehler et al., 2019)
	Zebrafish	*tyr*	- Melanin reduction levels.	(Jao et al., 2013)
	Medaka	*tyr*	- Amelanotic red eyes and melanin-free skin.	(Fang et al., 2018)
	White crucian carp	*tyr*	- Melanin reduction levels.	(Liu et al., 2019)
	Loach	*tyr*	- Production of albino mutants.	(Xu et al., 2019)
	Xenopus	*tyr*	- Melanin reduction levels.	(Nakayama et al., 2013)
Pigmentation	Oujiang color common carp	*mc1r*	- Melanin formation and albinism in the skin.	(Mandal et al., 2020)
		hps4	- Melanin biosynthesis diminution.	(Wang et al., 2022a)
	Tilapia	*csf1ra*	- Body color pattern formation.	(Lu et al., 2022b)
		tyrb	- Melanin biosynthesis reduction.	(Lu et al., 2022a)
	Cavefish	*oca2*	- Minimizing the mosaicism level.	(Klaassen et al., 2018)
	Yellow River carp	*oca2*	- Appearance of abnormal red skin color.	(Jiang et al., 2022)
Metabolism	Atlantic salmon	*srebp-1, Elovl2*	- PUFAs biosynthesis and lipid metabolism.	(Datsomor et al., 2019a; Datsomor et al., 2019b; Jin et al., 2020a)
	Channel catfish	*Elovl2*	- PUFAs biosynthesis improvement.	(Xing et al., 2022b)
	Zebrafish	*runx2b*	- Production of new strain devoid of IBs.	(Nie et al., 2022)
	Blunt snout bream	*runx2b*	- Production of new strain devoid of IBs.	(Dong et al., 2023)
	Gibel carp	*runx2b*	- Production of new strain devoid of IBs.	(Gan et al., 2023)
Intramuscular bones	Zebrafish	*bmp6*	- Production of new strain devoid of IBs.	(Xu et al., 2022)
	Crucian carp	*bmp6*	- Production of new strain devoid of IBs.	(Kuang et al., 2023)
	Zebrafish	*scxa*	- Reduction of 70% of IBs in adult mutants.	(Nie et al., 2022)

postnatal myogenesis and satellite cell growth via Pax7 signal (Liu et al., 2019) and impedes the overdevelopment through the activation of *Smad2/3* pathway to diminish the *MRFs* (Myogenic regulatory factors) expression (Dehler et al., 2016). Studies performed on different aquaculture species have offered several insightful perspectives on the *mstn* mutation and its key role in the modulation of cell proliferation and muscle growth. Unlike in freshwater species, few functional studies have been performed to generate CRISPR/Cas9-mediated disruption of *mstn* and grasp its effect on marine fishes. Accordingly, in red sea bream (*Pagrus major*) and olive flounder (*Paralichthys olivaceus*), a complete knockout of *mstn* led to an increase of 16% in skeletal muscle mass (Kishimoto et al., 2018), muscle hyperplasia, and body thickness in these two species, respectively (Kim et al., 2019). Per se, in freshwater fish such as common carp, the *mstn*-CRISPR/Cas9 mutated group showed a greater increase in the body weight and length with a remarkable augmentation in muscle cell number and fibers' size (Zhong et al., 2016), and double muscle phenotype in the produced F_0 without any abnormal effects (Shahi et al., 2022). In such situation, *mstn* KO in blunt snout bream (*Megalobrama amblycephala*) and loach (*Misgurnus anguillicaudatus*) resulted in a great augmentation in body weight and muscle development in Wuchang bream (Sun et al., 2020), and somatic growth with lipid accumulation in loach (Tao et al., 2021). Additionally, CRISPR/Cas9 *mstn* mutated F_4 medaka (*Oryzias latipes*) showed a significant increase in body weight and length (Yeh et al., 2017). In channel catfish (*Ictalurus punctatus*) and tilapia, *mstn* KO led to a significant increase in muscle hyperplasia, mean body weight (Khalil et al., 2017), and an improvement in the growth performance (Coogan et al., 2022; Wu et al., 2023). Notably, *mstn* disruption in the majority of the abovementioned species caused a positive effect on muscle growth without adverse impacts except for medaka, wherein, some mutant individuals appeared with some spinal deformities (scoliosis). Reportedly, in mice, *mstn* inhibition was postulated to interfere in the modulation of bone structure and formation (Bialek et al., 2014; Cui et al., 2020; Elkasrawy and Hamrick, 2010; Mendias et al., 2008). Even so, in zebrafish (*Danio rerio*), knockout of *pomc* (pro-opiomelanocortin) and *acvr2* (Activin A receptor, type II) induced muscle fibers development and enhanced growth performance (Che et al., 2023; Yang et al., 2023), whereas, mediating genetic mutation of *smyhc1* (slow fiber-specific myosin heavy chain 1) and *hsp90α1* (heat shock protein 90α1) caused inhibition of myosin thick filament formation in slow myofibers of embryos, hence arrest the organization of M-line in the sarcomere (Cai et al., 2018), and confirming their crucial functions in the these two key components of sarcomeres. In common carp, Zhong et al. (2016)

reported bone anomalies including bending back, opercula, and maxilla deficiency in *sp7*- CRISPR/Cas9 mutant group. Noteworthily, in mollusks, the potential application of this technology in Pacific oyster embryos targeting *MELC* (myosin essential light chain) disruption was performed, the resultant findings displayed a visible effect on larval muscle myogenesis and sarcomeric organization of thin filaments (Li et al., 2021a; Li et al., 2021b).

3.2. Reproduction

The harnessing of genome editing tools to produce populations that are desirable for the aquaculture industry and eco-friendly is promising. In this respect, functional studies have been undertaken with insertions or deletions of bases to investigate sex determination, sex reversal, germ cells, and fertility. These features have been broadly studied in zebrafish and tilapia targeting different causative genes, however, rare studies have been conducted in marine species. In fact, the objective of discovering master sex-determining genes in fishes is to come out with effective target genes that corroborate fish sex control breeding (Gratacap et al., 2019). Thus, the success of monosex individuals' acquisition via different editing tools has been achieved in several aquatic species through targeting site-specific genes including *Sry and dmy/dmrt1bY* in Japanese medaka (*Oryzias latipes*) (Matsuda et al., 2002; Nanda et al., 2002), *sdY* in rainbow trout (*Oncorhynchus mykiss*) (Yano et al., 2012), *amhy* in Patagonian pejerrey (*Odontesthes hatcheri*) (Hattori et al., 2012), *gsdf* in Nile tilapia (Jiang et al., 2016), and *dmrt1* in Chinese tongue sole (*Cynoglossus semilaevis*) (Cui et al., 2017). However, a sole study was performed on yellow catfish (*Pelteobagrus fulvidraco*) using CRISPR/Cas9 technology, where the results indicated a significant effect of *pfpdz1* gene in the modulation of male sex differentiation and maintenance (Dan et al., 2018). In contrast, several studies have been conducted to investigate sex reversal in fish via knocking out *gsdf* (Gonadal soma-derived factoris), *Sf-1*(nuclear receptor subfamily 5, group a, member 1), and *amhy* (anti-Mullerian hormone) genes in Nile tilapia (Jiang et al., 2016; Li et al., 2015; Xie et al., 2016), *Esr2a* and *Esr2b* genes in zebrafish (Lu et al., 2017) and *cyp17a1* gene in common carp (Zhai et al., 2022) leading to sex reversal from female to male in tilapia and zebrafish and production of all female carp. On the flipside, in terms of germ cell development, Lin et al. (2017) suggested *amh* and *dmrt1* key functions in the maintenance of the balance between germ cell proliferation and differentiation in males. In this regard, the generation of tilapia *dmrt6* male mutant resulted in a low level of 11-KT (11-ketotestosterone) and fewer spermatocytes at the initial stage (Zhang et al., 2014), whilst the *dmrt1* KO was reported to inhibit the transcription of *foxl3* in germ cells *and foxl2* and *cyp19a1a* in somatic cells, confirming the crucial role of *dmrt1* and *foxl3* in the regulation of sexual plasticity of germ cells (Dai et al., 2021). In its turn, the mutation of *nanos2* and *nanos3* (Nanos C2HC-Type Zinc Finger 2 and 3) and *piwil2* (piwi-like 2) genes led to primordial germ cells loss at the hatching stage in tilapia (Jin et al., 2020b; Li et al., 2014b). Per se, the dead-end (*dnd*) was reported to play a crucial role in the formation of the germ cell (Baloch et al., 2019), and KO of this gene during the beginning of embryogenesis engendered germ cell loss in medaka (Zhu et al., 2018) and in rainbow trout (Fujihara et al., 2022), however, the rescue of germ cells in Atlantic salmon (*Salmo salar*) *dnd* crispants resulted in the development of ovaries and testes after one year (Güralp et al., 2020). Additionally, studies dealing with the molecular bioprocess of fertility to produce transgenic populations with either fertile/sterile gonads via CRISPR/Cas9 tools are getting more attention recently. For instance, in tilapia, *foxh1* KO led to oogenesis arrest and female sterility without affecting males (Tao et al., 2020), meanwhile, *eEF1A1b* KO resulted in spermatogenesis arrest and a reduction in male fertility without any effect on females (Chen et al., 2017). Likewise, Zhang et al. (2020) reported insufficient spermatogenesis and a delay in ovary-to-testis transition in zebrafish *cyp11c1*−/− mutant males concomitant with egg spawning reduction and a defeat of germinal vesicle breakdown in female ones.

3.3. Disease resistance

To minimize negative impacts on aquatic animal welfare and cope with viral and invading pathogen infections, CRISPR/Cas9 system has been used frequently in recent years in order to enhance the immunity of organisms. In this respect, several studies have been performed on fish to produce strains more resistant to viral infection risks. For instance, Ma et al. (2018) knocked out the DNA sequence of the *JAM-A* (Junctional Adhesion Molecule-A) and assessed the resistance in-vitro against different GCRV (grass carp reovirus) genotypes, the findings demonstrated the successful utilization of this tool to generate grass carp (*Ctenopharyngodon idellus*) resistant to hemorrhagic disease caused by the latter. In accordance with this view, Chen et al. (2018) attempted to explore and estimate the role of *itgb1b* (Integrin - β1) during the entrance of viruses. The obtained data showed *itgb1b* mutant grass carp had a great response in the limitation of viral entry into the cells through clathrin. Similarly, Zhu and his co-workers generated *prmt7*-null zebrafish and subjected them to SVCV (spring viremia of carp virus) and GCRV (grass carp reovirus). The results evinced that mutated groups were viable and similar to wild-type group in terms of reproductive ability and development. However, they were more resistant to viral infection compared to wild type siblings (Zhu et al., 2020). To some extent, in olive flounder, Kim et al. (2021) isolated and characterized *Maf1* (a negative modulator of RNA polymerase III- dependent transcription) gene to explore its role in viral infection response, at the termination of the experiment they concluded that *Maf1* disruption led to an augmentation in VHSV (viral hemorrhagic septicemia virus) glycoprotein (G) mRNA levels suggesting its subtle role in the immune response when this kind of infection occurred. In another aspect, Simora et al. inserted alligator cathelicidin gene in a genome non-coding region of channel catfish. They recorded a high integration rate of this gene with high expression levels in the blood and gill as well as in other tissues (Simora et al., 2020). Nevertheless, they reported increasing embryo mortality when they augmented the mutation rate. In such situation, an early study was performed to generate transgenic blue catfish (*Ictalurus furcatus*) in site-specific knock-in of the *As-Cath* (alligator cathelicidin) gene, the results demonstrated a great integration efficiency and an improvement in disease resistance in *As-Cath* transgenic fish (Wang et al., 2023). It is worth noting that, in-vitro studies in aquatic animals targeting disease resistance to improve fish welfare and health are very scarce. In this matter, Dehler et al. (2019) used CRISPR/Cas9 to knock out *stat2* (signal transducer and activator of transcription) in Chinook salmon (*Oncorhynchus tshawytscha*) cell line investigating its function. The findings showed production of EHNV (epizootic hematopoietic necrosis virus) DNA viral particles and VHSV (viral hemorrhagic septicemia virus) RNA, indicating the role of *stat2* gene in the ability to resist a viral infection.

3.4. Pigmentation

Based on their function, pigmentation genes can be classified into pigment cell interaction or pigment cell differentiation and migration in teleost fish. Thus, several kinds of pigments have been studied in aquatic animals including melanin, carotenoids, pteridine, and guanine platelets (Wang et al., 2021a). From these pigments, melanin continues to fascinate considerable and increasing attention from researchers with a focus on possible genes that may interfere directly or indirectly to modulate the molecular mechanism of this pigment. To understand the melanin biosynthesis pathway and its modulating signal through *tyr* (tyrosinase) gene and its homologous genes in fishes, a previous study was undertaken using the CRISPR/Cas9 system to knockout *tyr* in medaka, this mutation led to amelanotic red eyes and melanin-free skin (Fang et al., 2018). Similarly, *tyr* KO in zebrafish, white crucian carp (*Carassius auratus cuvieri*, WCC) which is a subspecies of goldfish, Xenopus (*Xenopus tropicalis*), and loach resulted in melanin reduction levels (Chen et al., 2019; Edvardsen et al., 2014; Jao et al., 2013; Liu et al., 2019;

Nakayama et al., 2013; Xu et al., 2019). Furthermore, in Oujiang color common carp (*Cyprinus carpio* var. color), *mc1r* knockout mutants appeared with grayish skin color instead of black and reduced melanin formation with albinism appearance in the skin when they were subjected to low and high mutation rates, respectively (Mandal et al., 2020). On the other hand, to elucidate and confirm the crucial role of *oca2* (oculocutaneous albinism II) gene in the molecular mechanism of pigmentation, two studies were conducted in cavefish (*Astyanax mexicanus*) (Klaassen et al., 2018) and Yellow River carp (*Cyprinus carpio haematopterus*) (Jiang et al., 2022) using CRISPR/Cas9 approach. The findings indicated that this gene is a key modulator of albinism evolution in cavefish populations and appearance of the abnormal red skin color in Yellow River carp. Noteworthily, in tilapia, several studies were performed to understand and improve the pigmentation trait through this editing tool. For instance, the disruption of *hps4* (Hermansky-Pudlak Syndrome 4) and *tyrb* led to melanin biosynthesis diminution and complete disappearance of black blotches from the skin of GMT (genetically male red tilapia), respectively (Lu et al., 2022a; Wang et al., 2022a). Moreover, the homozygous mutation of *pmela and pmelb* (Pre-melanosome protein a and b) led to the reduction of melanophores (in number and size) which caused yellowish body color and hypo-pigmented retinal epithelium. However, the high mutation rate (double) led to the loss of more melanophores and increase of xanthophores (in number and size), thereby producing individuals with golden body color (Wang et al., 2022b). Accordingly, Wang et al. (2021a) suggested that tilapia is an excellent model system to understand the color pattern mechanisms and the formation of body color in cichlids.

3.5. Metabolism

Studies broached the metabolism trait in aquaculture species via the harnessing of gene-editing tools such as CRISPR/Cas9 are scarce and limited. So forth, researchers from Norway and USA attempted to identify and understand polyunsaturated fatty acids (PUFAs) biosynthesis in fish due to their importance and benefits for human health and immunity. In this regard, Datsomor et al. (2019a) performed a feeding trial for 54 days with CRISPR-mutated Atlantic salmon (Δ6abc/5^{Mt} and Δ6bc^{Mt}) that were fed with low and high LC-PUFA diets. They suggested that *Srebp-1* (sterol regulatory element binding protein-1) is a major transcription regulator of salmon LC-PUFA biosynthesis. Likewise, they generated *elovl2* knock-out (very long-chain fatty acyl elongase) to investigate its effect on LC-PUFA biosynthesis. The results demonstrated a diminution in DHA (docosahexaenoic acid) (which is one LC-PUFA) levels and accumulation of the latter in the liver, brain, and white muscle (Datsomor et al., 2019b). In line with these reports, Xing et al. (2022b) successfully integrated the masu salmon (*Oncorhynchus masou*) *elovl2* gene into channel catfish genome with high rates which cause an increase of DHA content and total n-3 PUFAs. These results provided valuable information on the application of CRISPR/Cas9 in aquatic animal nutrition research and bridge the gap between this field and genomic selection to enhance this trait of interest, moreover, the adoption of GWAS in this research area will underpin and support the determination of more causative genes hence subjecting them to mutagenesis manipulation in order to produce sustainable and cost-effective feeds or populations with high content of omega-3 fatty acids.

3.6. Intermuscular bones

Intermuscular bones (IBs) are fine spicule-like bones with almost tierce to one-half of the rib's length distributed ordinarily in the fish from the head to tail (Li et al., 2013). These small structures lead to multiple impacts on the processing options, food value, and possible injury or trauma if lodged in the throat or mouth (Knight and Lesser, 1989; Lin et al., 2014; Nie et al., 2020). In teleost lineages such as cyprinids, IBs range from 73 to 169 bones and are characterized by extreme complexity in their morphology (Yang et al., 2019). Recently, benefiting from sequencing technologies to identify genetic loci and genes related to IBs, the production of IBs-free mutants is a hot concern that has been and will continue to attract considerable and increasing attention from researchers. In this respect, earlier studies have been conducted in cyprinids by targeting different causative genes. For instance, the harnessing of CRISPR/Cas9 to generate *runx2b* (runt-related transcription factor 2b) mutant zebrafish, blunt snout bream, and gibel carp (*Carassius gibelio*) led to the production of new strains completely devoid of IBs without jeopardizing the mineralization of other bones and muscles nutritional composition (Dong et al., 2023; Gan et al., 2023; Nie et al., 2022). Similarly, *bmp6* (bone morphogenetic protein *6*) KO in zebrafish and crucian carp (*Carassius auratus*) resulted in IBs-free populations without any adverse effects on growth and bone changes (Kuang et al., 2023; Xu et al., 2022). To some extent, Nie et al. (2022) performed another trial in zebrafish using the same technology to knock out *scxa* (*scleraxis a*) gene and investigate its effect, the findings demonstrated a reduction of 70% of IBs in adult mutants. However, the ribs were significantly affected. It is worth noting that, the production of new IBs-free strains via genome editing tools is an important breakthrough compared to various processing technologies. However, it is still in its infancy and further research is needed for mining more candidate genes associated with this trait as well as targeting other aquatic species.

4. Challenges and perspectives

CRISPR/Cas9 approach was reported to be an efficient and robust technique to manipulate genes in finfish, crustaceans, and mollusks (Chakrapani et al., 2016; Dehler et al., 2016; Gratacap et al., 2020; Hamar and Kültz, 2021; Irion et al., 2014; Li et al., 2021b; Li et al., 2019; Liu et al., 2019; Ou et al., 2023; Zhong et al., 2016). Technically, the microinjection was reported to be an effective method to transfect CRISPR/Cas9 into the egg or embryo of aquatic animals and generate mutagenesis. Nonetheless, it has been claimed to be inappropriate for some fish species' eggs due to many factors such as the nature of the yolk cell, perivitelline space width, chorion hardness and thickness, its difficulty of removing from the egg (Goto et al., 2019). To overwhelm these pitfalls, different protocols (alignment-based and scoring-based methods) and computational approaches (such as CHOPCHOP, CRISPR RGEN tools, and CRISPOR web platforms) have been adopted to design expression vectors for Cas9 protein and sgRNA to increase their expression effectiveness. These approaches have been reviewed in detail elsewhere (Liu et al., 2020; Potts et al., 2021; Wang et al., 2021b). Moreover, high fecundity and small size of eggs, immobilization and arrangement of eggs, the diameter of the needle, and time-consuming (limited number of injected cells) are other limiting factors that have been reported in marine invertebrates such as Pacific oyster (Yu et al., 2019), sea urchin (*Strongylocentrotus purpuratus*) (Stepicheva and Song, 2014), and lancelet (*Branchiostoma lanceolatum*) (Hirsinger et al., 2015). In this regard, Robert and his colleagues proposed a mass delivery of CRISPR/Cas9 through the electroporation technique, natural transfection, or adopting another approach already used in chicken wherein the complex CRISPR/Cas9 was delivered via sperm to manipulate the fertilized eggs (Lars and Beata, 2022). In another aspect, the application of this system to enhance complex traits in aquaculture species appeared with some downsides or pleiotropic effects in certain species such as bone anomalies in *sp7a*-CRISPR mutated F_0 carps, a spinal deformity (scoliosis) in some *mstn* CRISPR mutated medaka, a heaviest embryo mortality after the injection of alligator cathelicidin gene in channel catfish, and high mosaicism in *piwil2* KO F0 tilapia. These abnormalities urge the need for the standardization of updated and/or new species-specific protocols more robust and highly efficient benefiting from the progress of nanoscience and advances in genotyping and sequencing technologies such as CRISPR/Cas12a, CRISPR/Cas13a, and CRISPR/Cas biosensor. In tandem, the scientific progress in genomics and bioinformatics research will offer more opportunities for researchers to explore and generate mutagenesis in marine farmed fish, where we could

retrieve a few functional studies that have been conducted in these animals such as Chinese tongue sole, olive flounder, red sea bream and large yellow croaker (*Larimichthys crocea*). This could, in turn, be ascribed to the solid chorion that protects embryos and the high internal pressure of eggs which may lead to backflow of egg material and damage of the glass micropipette tip (Goto et al., 2019). On the other hand, the difficulty of adjusting and keeping up the animal pole of the egg needed for microinjection due to the pelagic feature of the eggs (buoyancy) produced by the majority of marine fishes (Li et al., 2023). Penultimately, using this technology in future research is a prerequisite to improve and explore other traits of interest such as the metabolic process of protein deposition and glucose metabolism, nutritional value, meat quality, and environmental stressors tolerance. Additionally, it may come out with insightful knowledge in different research areas including aquatic animal nutrition and environmental studies. Finally, regulatory framework approval, risk assessment, and consumer acceptance are other factors that needed to be taken into consideration for the commercialization of CRISPR technology products (Okoli et al., 2022).

In conclusion, the application of CRISPR/Cas9 system in aquaculture science is promising and has given more insightful knowledge to improve different economic traits. Moreover, benefiting from recent approaches and technologies such as high throughput sequencing, omics-based technologies, and genome-wide association studies may assist to discover more key genes associated with different complex traits in freshwater and marine species including crustaceans, thus paving the way for more functional studies to ameliorate the genetic breeding programs and boost the aquaculture production.

Author contributions

AM wrote the paper; SL supervised and revised the manuscript.

CRediT authorship contribution statement

Ahmed Mokrani: Investigation, Formal analysis, Writing – original draft. **Shikai Liu:** Supervision, Conceptualization, Resources, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest to report.

Data availability

No data was used for the research described in the article.

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