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REVIEW

REVIEWS IN Aquaculture

# Optimising commercial traits through gene editing in aquaculture: Strategies for accelerating genetic improvement

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## Abstract

Aquaculture is one of the fastest-growing food production sectors. As the global human population continues to increase and further pressure is added to the prospects of achieving global food security, aquaculture is expected to play an integral role in meeting future nutrition demands. With advances in genetic technologies over recent years, much progress has been made within the realm of selective breeding. Despite success, selective breeding programs have limitations to the rate of genetic gain they can achieve. The incorporation of targeted genetic technologies, such as gene editing, into research related to selective breeding programs will help identify specific genes related to commercially desirable traits, as well as expedite genetic improvement. This review summarises research encompassing the most commonly targeted traits using gene editing within aquaculture, namely reproduction and development, pigmentation, growth and disease resistance. In addition, this review illustrates how the incorporation of gene editing can expedite genetic improvement through the rapid fixation of desirable alleles, as well as suggests strategies to accelerate genetic improvement for aquaculture production.

**KEYWORDS**
aquaculture, CRISPR, gene editing, genome, selection, traits

## 1 | INTRODUCTION

The human population is expected to reach 10 billion people by the year 2059,[1] which will have profound impacts on the hopes of achieving food security on a global scale. In addition, other stressors, such as climate change, catastrophic weather events and limited natural resources, are further complicating efforts to achieve food security. Increased demand for housing development will coincide with the increased demand for high-quality protein, creating competition for agricultural land use.[2] This competition will drive more efficient and sustainable animal production systems. As a result, aquaculture is expected to play an important role in meeting future nutrition demands. In terms of feed conversion and protein retention, aquaculture is considered to be very efficient and comparable, or even better depending on the species, to most livestock.[3] The seafood industry serves as a primary source of protein for numerous populations around the world, as well as a provider of employment.[4] Seafood products provide a major source of long-chain polyunsaturated fatty acids, which are an essential component of a healthy diet.[5] Putting a greater emphasis on aquaculture production will help supplement protein sources and address concerns over global food security.

Aquaculture is one of the fastest growing sectors of food production.[6] Currently, aquaculture production rivals that of capture fisheries; in the year 2020, capture fisheries contributed 51% of total production compared to aquaculture contributing 49%. Furthermore, according to the Food and Agriculture Organisation's (FAO) recent

report on the state of world fisheries and aquaculture, aquaculture is expected to grow 31% over the next 10 years.[6] Presently, most aquaculture production occurs inland, in freshwater, and in nearshore marine waters,[7] with the majority of finfish aquaculture occurring inland.[6] In fact, the average production of inland aquaculture has more than doubled within the last 20 years.[6] The potential for growth within the industry is very high given that only 1% of suitable marine areas for aquaculture are currently being utilised,[8] and there are many more species in aquaculture compared to terrestrial livestock. As of 2022, the FAO has estimated 652 different species items making up aquaculture production.[6] Additionally, in comparison to terrestrial livestock and crop sectors, aquaculture species are in the early stages of the domestication process.[9] This means that there is higher genetic diversity within-species and leaves room to select for specific traits out of a diverse gene pool that will better address the challenges that the industry is currently facing (i.e., disease, slow growth, and reduction of fertility in production systems).[10]

Selective breeding is the process of choosing breeding candidates to increase the occurrence of desired phenotypic, physiological, morphological and/or behavioural traits in a population through the accumulation of advantageous alleles. Some of the most commonly targeted traits in aquaculture include those associated with growth, disease resistance, reproduction and development, and pigmentation.[11] Phenotypic records and pedigrees, along with the heritability of each trait, shape the estimation of individual breeding values upon which selective breeding has traditionally been established. However, the process of breeding for a specific trait can be often slow, especially if the heritability underlying a desired trait in the population under selection is low. The extensive use of genomic resources, such as genotyping by sequencing, whole genome reference sequences, and high-density SNP genotyping arrays, in artificial selection and domestication in aquaculture is a relatively recent phenomenon.[12–16] The improvement of genomic technologies and approaches have allowed for more efficient identification and selection of animals carrying desired combinations of alleles.[17] Due to the naturally high fecundity of aquatic species and their associated large nuclear families, there is a high potential for stringent selection intensity and genetic gain. Breeding programs are able to utilise the naturally high genetic diversity found in wild populations when pursuing potentially beneficial phenotypes for production.[9]

A key factor in selective breeding is the ability to determine whether the genetic variation within target traits is the result of major-effect loci or numerous loci of a minor effect.[9] Traits controlled by a single gene, such as sex determination in medaka (*Oryzias latipes*),[18,19] can easily be selected. However, most traits that play a significant role in evolution or aquaculture production are often complex traits, making the identification of the multiple genes involved in the variation of a trait a difficult task.[20] Genome-wide association studies are often conducted to help determine genetic variations or markers associated with a specific trait. Once associated with desired traits, the markers can be used to help select broodstock for breeding, also known as marker-assisted selection (MAS).[17] Fine-scale quantitative trait mapping can help determine which genes are responsible for desired traits and has been proven to be a valuable tool for several traits in selective breeding.[21–23] More recently, genomic selection (GS) has shown great promise for incorporation within breeding programs. By simultaneously incorporating a large number of markers across the genome, GS is able to better explain genetic variation in relation to certain traits as well as better estimate genetic relatedness among individuals through the recognition of linkage disequilibrium between single nucleotide polymorphisms (SNPs) and quantitative trait loci (QTL). On average, GS has been shown to increase the prediction accuracy by 22% for disease-resistance traits and 24% for growth-related traits.[9]

Even with the current state of innovative technology and genomic screening approaches, there are several shortcomings in regard to current selective breeding programs in aquaculture. First, the genomes of numerous aquaculture species have yet to be sequenced and assembled.[10] Another confounding factor is the low heritability of many target traits (i.e., disease resistance), often due to epigenetic effects or numerous loci affecting a single trait.[24] It is expensive to generate vast numbers of full- and half-sib families to measure and record phenotypes, and the generation interval of some species further impedes the efficiency of this goal.[9] Furthermore, most breeding programs select for multiple target traits at one time in order to maximise profits and speed up the domestication process, making it more difficult to exploit completely the heritability of a single trait. It is also difficult to determine the effects of different environmental conditions, as well as their influence on how particular genotypes may be expressed.[25] Quantifying these effects (i.e., genotype by environment interactions) should also be an important consideration within breeding programs. Another difficulty is the management of inbreeding within closed breeding populations. Having a closed system limits genetic variation in broodstock and any mutations that may arise are often novel.[9]

The incorporation of gene editing can help identify specific genes related to commercially desirable traits and has the potential to expedite genetic improvement through the rapid fixation of desirable alleles.[26] As the utilisation of gene editing technologies, such as zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced palindromic repeats (CRISPR), have been adapted for aquatic species, much progress has been made in the identification of genes that influence certain valuable production traits. However, further research is necessary to produce well-developed genomes for commercially important species in order to help identify functional genes influencing desirable traits and to further develop gene editing technologies in lesser-researched aquatic species. The purpose of this review is to summarise past research encompassing the most commonly targeted traits within aquaculture, illustrate how the incorporation of gene editing in the aquaculture industry can expedite genetic improvement through the rapid fixation of desirable alleles, as well as suggest strategies to accelerate genetic improvement for aquaculture production.

## 2 | GENE EDITING

There are two broad types of genetic modification technologies: gene transfer and gene editing. The first production of a genetically

modified fish was accomplished through gene transfer using microinjection in goldfish (*Carassius auratus*) embryos.[27] Subsequently, other gene transfer applications were applied in aquatic species such as electroporation,[28] virus-mediated transfer,[29] sperm-mediated transfer,[30] as well as transposon-mediated transfer.[31] Classical methods of gene transfer relied on the random integration of the vector into the genome, potentially leading to unpredictable consequences such as the possible insertional mutagenesis of host genes and rearrangements of the transgene.[32] Alternatively, gene editing has become increasingly popular across many realms of science. Genome editing is broadly defined as the intentional modification of an organism's genome, often resulting in either an insertion, deletion or replacement within any region of the genome; narrowly defined, gene editing refers to editing occurring only within the coding region of the genome.[33] Gene editing allows for the study and alteration of gene function. The development of engineered nucleases has allowed for precise gene editing of a target sequence. Compared to traditional methods of gene transfer, the application of engineered nucleases are highly specific, easier to design, more efficient and have fewer off-targets.[34] Gene editing technologies, such as ZFN, TALEN and CRISPR, have been applied to develop genetically edited organisms (Figure 1). The development of gene editing technologies

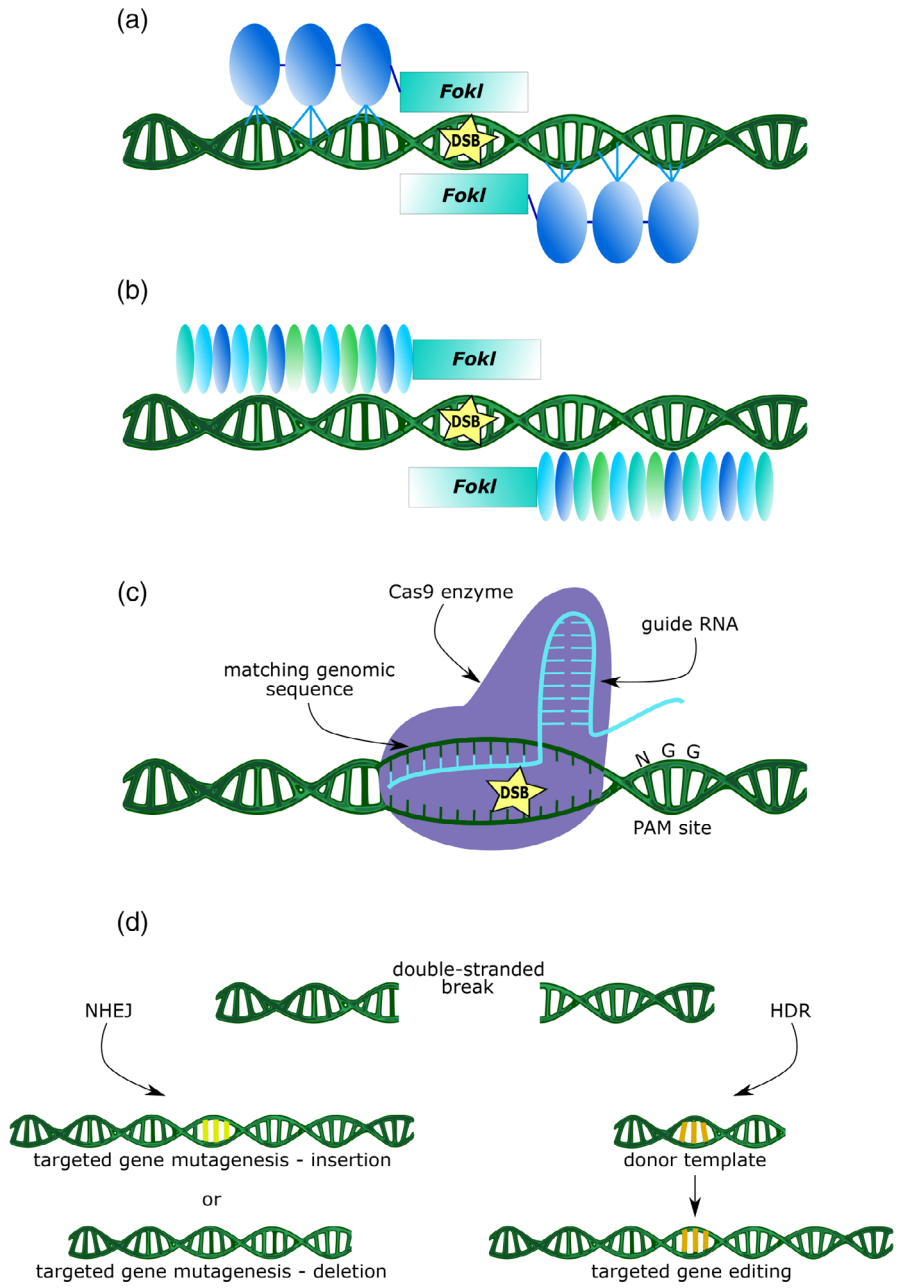


**FIGURE 1** Schematic diagrams of gene editing technologies (a) zinc-finger nucleases (ZFN), (b) transcription activator-like effector nucleases (TALEN), (c) clustered regularly interspaced palindromic repeats (CRISPR) and (d) double-stranded break repaired through nonhomologous end joining (NHEJ) or homology-directed repair (HDR). DSB, double-stranded break.

has greatly improved the feasibility and efficacy of producing genetically modified fishes for aquaculture production.

The mechanisms of gene editing can be broken into three steps: recognition, cleavage, and repair.[35] Once the nuclease recognises the target sequence for the gene of interest, the targeted site is cleaved, creating a double-stranded break. The double-stranded break is then repaired through either nonhomologous end joining (NHEJ) or homology-directed repair (HDR).[36] In the absence of a repair template when reconstructing a double-stranded break, NHEJ indiscriminately ligates the DNA back together with minimal reference to the DNA sequence. This imprecise repair can lead to nucleotide insertions or deletions that result in frameshifts, premature termination codons, and loss of gene function through nonsense-mediated decay. Therefore, NHEJ repair is often utilised to knockout, or inactivate, a gene of interest. Alternatively, HDR requires the incorporation of a sequence that is highly similar to the severed and intact donor strand of DNA. HDR is often used to knock-in genes by flanking the insertion sequence with homology arms, or regions with flanking homology to the double-stranded site, with the knock-in sequence located between the homology arms[36,37] (Figure 1).

Gene editing can be used for gene knockout, gene knock-in, gene knock-up, as well as gene knockdown.[38] High throughput loss of function gene editing is useful for identifying gene function, especially for target traits where annotation of gene function is elusive, or for organisms that have a general lack of gene function data. In fact, implementing the knockout or knockdown of a certain gene in order to respectively reduce or eliminate its function allows its role in physiological or pathological processes to be elucidated through phenotypic measurements and observations.[39,40] Germline gene editing is often conducted within the early stages of embryonic development so that modifications are incorporated into progenitor germ cells that develop further to produce gametes, enabling modifications to be passed down to future generations.[41] The knockout of genes can be accomplished with the aforementioned gene editing technologies such as ZFN, TALEN and CRISPR, while the knockdown of genes, is usually accomplished through RNA interference (RNAi) and/or morpholino antisense oligonucleotide knockdown. While RNAi works to reduce the level of a gene product, morpholino antisense oligonucleotides work by altering mRNA metabolism or translation.[42,43] However, it is important to note that the knockdown of a gene usually only results in partial loss of function (LOF); thus, some protein functions may still remain. Nevertheless, RNAi may be a useful technique when standard gene editing technologies are not plausible or in cases where a full knockout is not necessary. Therefore, gene editing technologies that implement gene knockout are much more popular for LOF studies, as well as to accelerate genetic improvement. The knock-in of genes involves the introduction of an exogenous DNA fragment at a specified site. So far, the knock-in of genes is not commonly used in aquaculture since this technique is more challenging. Additionally, political, and public perception of knock-in gene technology in the food sector is not as favourable and is sometimes prohibited depending upon the country.[44] This review will primarily focus on genomic technologies capable of accelerating genetic improvement through the cumulation of permanent and heritable mutations.

## 2.1 | Zinc-finger nucleases

ZFN are artificially engineered endonucleases composed of DNA-recognition domains and non-specific endonucleases.[45,46] The DNA-recognition domains contain three or more $C_2H_2$ zinc finger motifs, while the non-specific endonuclease is a type IIS restriction enzyme, *FokI*. DNA-recognition domains are engineered to target specific regions of DNA sequences, while the non-specific endonucleases perform cleavage[47] (Figure 1). When ZFN introduce double-stranded breaks, the repair is either through NHEJ or HDR. ZFN technology was initiated in 1996, with its popularity rising from 2003.[48] The majority of work involving ZFN in aquaculture-related species has been for LOF or gene knockout studies.[49]

ZFN technology was first utilised in teleosts through gene editing experiments focusing on mutagenesis in zebrafish (*Danio rerio*).[50,51] Initially, studies focused on achieving LOF of strikingly evident phenotypes to validate methodology and technologies. For example, Doyon et al. targeted the golden (*Slc24a5*) and no tail/Brachyury (*Ntl*) genes in zebrafish[50] and Ansai et al. targeted exogenous *EGFP* genes in medaka (*Oryzias latipes*).[52] After the validation of these methodologies, studies utilising ZFN-based gene editing continued to focus on gene knockout. The majority of these studies related to aquaculture species focused on targeting traits for reproduction and development[53–56] (Figure 2). However, despite advances made in ZFN editing technologies over the years, it remains a difficult, time-consuming and expensive technology[57,58] and has been shown to introduce off-target effects and therefore not widely applied to aquaculture species.[59]

## 2.2 | Transcription activator-like effector nucleases

The second most popular gene editing technology is transcription activator-like effector nucleases (TALEN). TALEN technology was first introduced around 2009, with its popularity increasing shortly after.[48] TALEN contains a DNA-recognition domain known as the transcriptional activator-like effector, as well as an endonuclease, most often *FokI*.[60] Similar to ZFN, the TALEN technology has been engineered to produce double-stranded breaks that are repaired through NHEJ or HDR. However, compared to ZFN, TALEN has a higher degree of accuracy and an expanded editing scope.[61,62] While ZFN is limited to identifying three bases per template along with a short DNA-binding sequence, TALEN is capable of identifying only one base and the DNA-binding sequences can be longer than 18 bp, thereby increasing targeting options[60] (Figure 1). Modification of the transcription activator-like effectors allows for a variety of targeting sequences.[63]

Similar to ZFN, studies incorporating TALEN in aquaculture species have mostly focused on LOF through gene knockout. Before CRISPR, TALEN technology was popular and frequently used since it is much easier to design components of TALEN compared to ZFN (Figure 3). However, TALEN are only able to target one site at a time, are difficult to engineer, and are unable to cleave methylated DNA.[64]

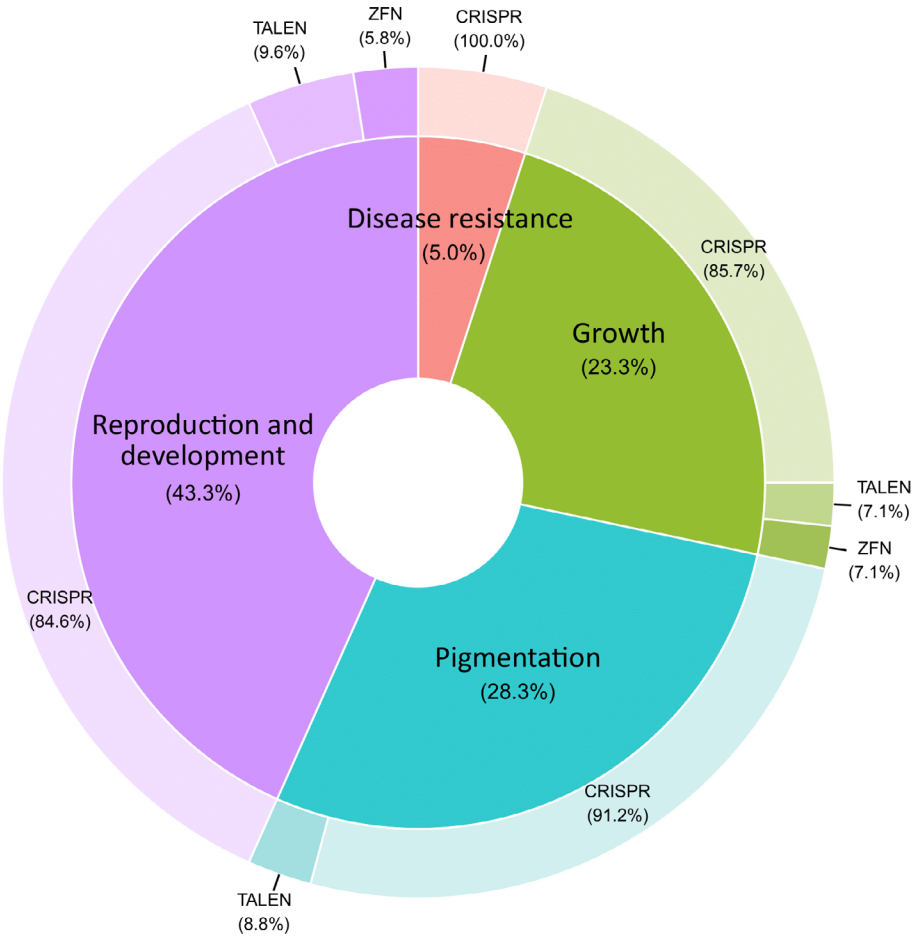


**FIGURE 2** Summary of the most commonly targeted traits relevant to gene editing in aquaculture species and the gene editing technologies those studies used. Model species were excluded from this figure.

## 2.3 | Clustered regularly interspaced palindromic repeats/Cas9

The most recent and popular gene editing technology is the CRISPR system. CRISPR gene editing began around 2012, and its usage continues to grow in popularity across many realms of biology[48] (Figure 2). The CRISPR/Cas system can be divided into two main classes, with the classes being further subdivided into 6 types and 33 subtypes.[65] However, the majority of CRISPR studies utilise the CRISPR/Cas9 system, which is composed of a Cas9 protein responsible for target DNA cleavage and a single-guide RNA (sgRNA)[48,66] (Figure 1). The sgRNA is made up of CRISPR RNA (crRNA), a 17–20 nucleotide sequence that is identical to the target DNA, and trans-activating crRNA (tracrRNA) which acts as a binding scaffold for the Cas nuclease.[67] The sgRNA is site-specific and is located upstream of a PAM-site which contains the DNA motif '-NGG-'. The PAM site, also known as the protospacer adjacent motif, is a short sequence of DNA that is usually about 2–6 base pairs downstream of the targeted DNA sequence. The Cas9 protein cuts 3–4 nucleotides upstream of the PAM site.[66] In some cases, a recombinant vector encoding the sgRNA sequence and Cas9 protein can be directly transfected into cells for gene editing.[68] For the vector-based CRISPR/Cas9 system, an RNA polymerase (Pol III) promoter like H1 or U6 is typically used for gRNA production, while a Pol II promoter is used for Cas9 expression.[69,70] Insufficient guide RNA expression may require the use of a different promoter or the use of gRNA/Cas9 protein ribonucleoprotein (RNP) complexes.[71] Similar to ZFN and TALEN, the double-stranded breaks are repaired by NHEJ or HDR.

The CRISPR/Cas9 system has been applied to numerous aquaculture species. A variety of microinjection and transfer platforms are available to deliver the recombinant vector into fertilised eggs at the one-cell stage.[72] Other methods of delivering the recombinant vector include electroporation, lentivirus-mediation, or the use of ribonucleoprotein complexes (RNP).[73–75] Several online software programs are available to design the sgRNA, pinpoint possible off-target effects, and predict the efficiency of the designed sgRNA.[76] Relative to TALEN and ZFN, CRISPR has a much higher efficiency, and is much easier to design and operate.[77,78] This system has the advantage that it is able to easily target multiple genes at one time and can significantly shorten the amount of time it takes to introduce targeted traits compared to traditional selective breeding techniques involving crossing and backcrossing,[9] making CRISPR/Cas9 the most preferred of the existing gene editing tools.[78]

Within the past few years, the use of the CRISPR/Cas9 system within aquaculture research has greatly expanded (Figure 3) and has

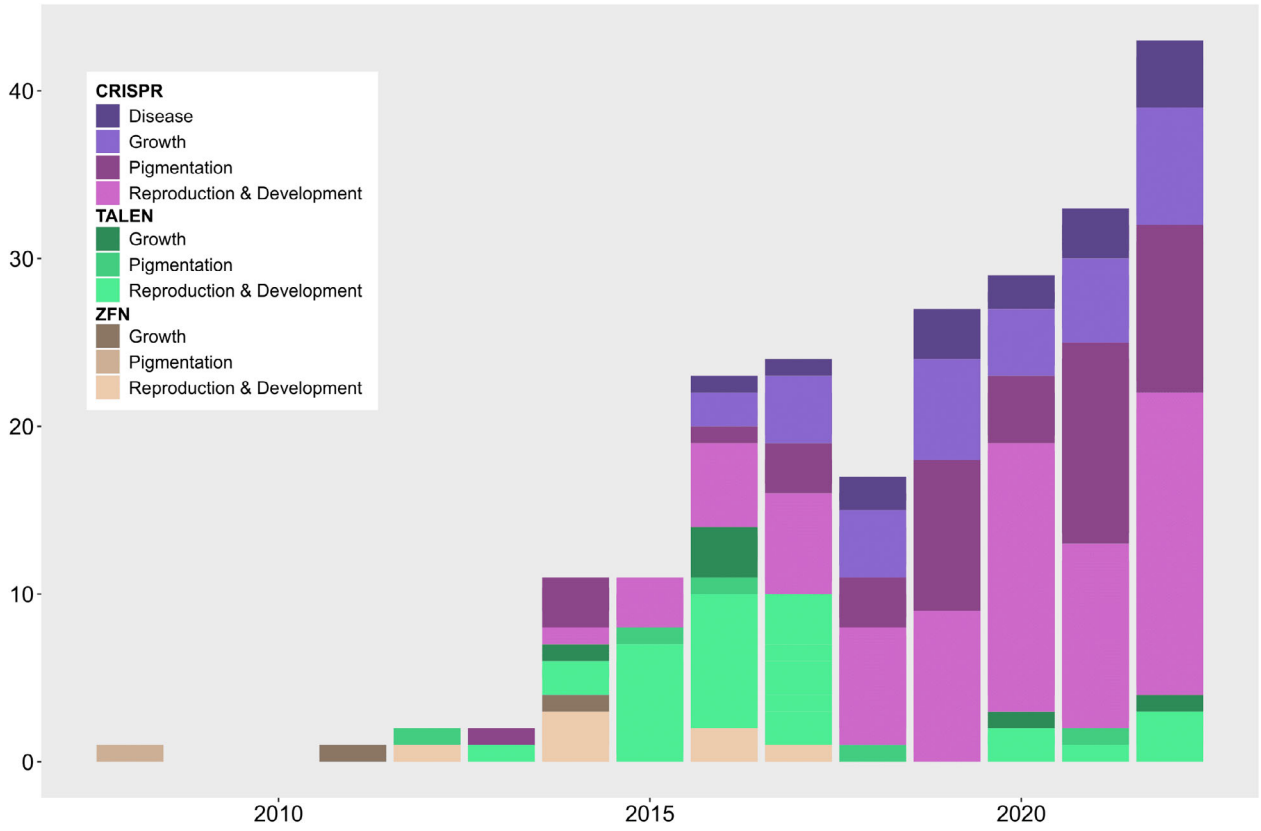


**FIGURE 3** Changes in the use of gene editing technologies in aquaculture-related studies between the years 2008–2022.

also proven to be a popular tool in cell line research.[79–81] Despite all of the benefits the CRISPR/Cas9 system provides, there are a few disadvantages. The CRISPR/Cas9 system can sometimes produce off-target effects and/or cleave in nontarget sites that have similar sequences to the target site.[82–84] Other challenges may include ancestral whole-genome duplication and mosaicism.[85–91] Mosaicism occurs when CRISPR/Cas nucleases retain activity after the first event of embryo cleavage, resulting in varying combinations of mutations among cell lineages.[92] All teleosts went through three rounds of genome duplication throughout vertebrate evolution. In fact, 2–6 rounds of differential genome duplication may have occurred among phylogenetic lineages of fishes.[93] Unlike terrestrial livestock species, ancestral whole-genome duplication can present several complications when it comes to gene editing in finfish due to the potential of having multiple copies of the same gene within a single organism, with each copy of gene not always fulfilling the same role.[94] Therefore, the effects of ancestral whole-genome duplication must be considered when designing target sequences for gene editing. Further challenges for the application of gene editing within aquatic species include the lack of an adequate gene editing platform for some fish with a tough chorion, lack of clarification for trait-related genes, difficulty detecting off-target effects in organisms without a sequenced genome and a general lack of protocol for understudied species.[10] Despite these challenges, scientists have been able to utilise this technology to work towards addressing several of the major issues within the aquaculture industry, which will be further discussed within the next section.

## 3 | SUMMARY OF CURRENT RESEARCH

This review compiled studies focusing on commonly targeted traits for gene editing relevant to aquaculture species; namely, those associated with reproduction and development, pigmentation, growth and disease resistance.[85] Any study involving the investigation of a gene that may be applicable to the aquaculture industry was included in this review. Google scholar (GS) and Web of Science (WoS) were used as databases for scholarly article selection. First GS was used to create an initial list of studies, then WoS was used to ensure that there were not any articles overlooked. Search strings were used to incorporate relevant terms such as gene editing, aquaculture, fish, CRISPR, TALEN, ZFN, reproduction, development, growth, pigmentation and disease resistance (Figure A1). This search included articles from 1995 to 2022, for the incorporation of all gene editing technologies briefly introduced in the previous section. The title of an article was initially inspected, then the abstract and method section were further screened for inclusion. Exclusion criteria, such as Master theses, or magazine articles, were applied (Figure B1).

In terms of species composition for each commonly targeted trait, species from Cyprinidae, Cichlidae and Adrianichthyidae, made up a

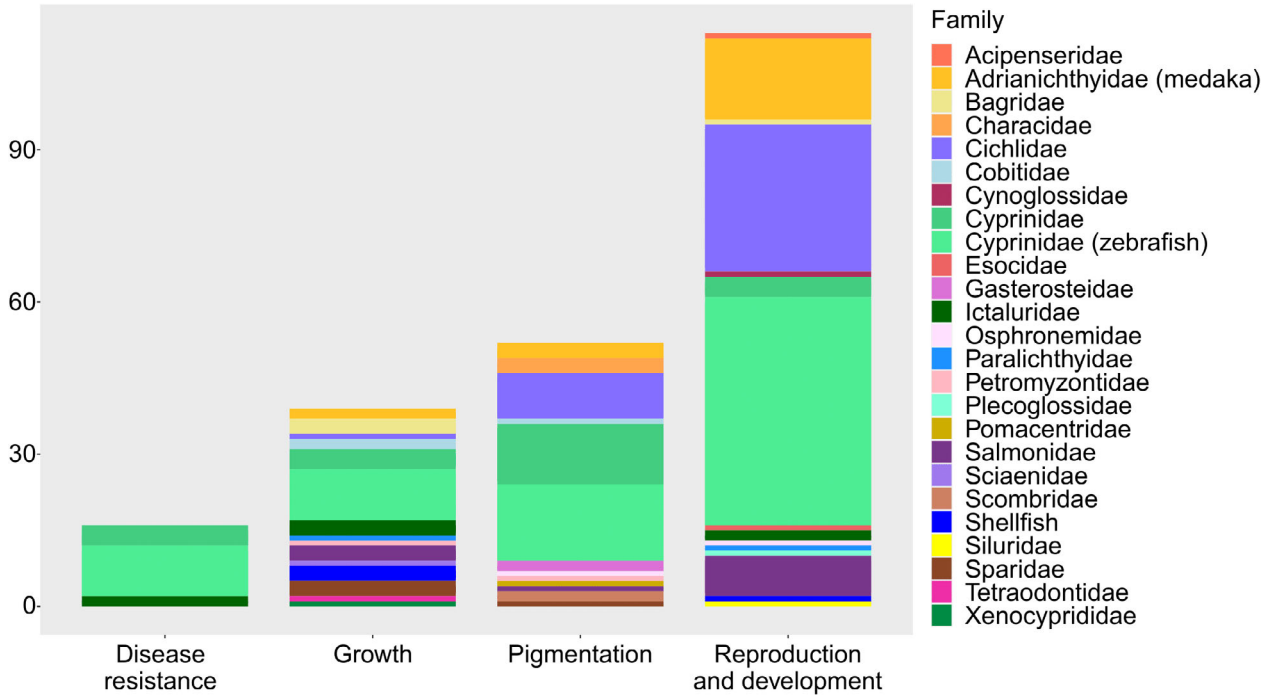


**FIGURE 4** The distribution of species (at the level of Family) found in the literature search for the most commonly targeted traits relevant to gene editing in aquaculture species. The *x*-axis depicts the targeted trait while the *y*-axis depicts the total number found in the literature search.

large portion of the total studies, most likely due to a considerable focus on zebrafish, tilapia, medaka and carp (Figure 4). Even though zebrafish are not considered an aquaculture species, they were included in this review due to the fact that zebrafish serve as a model species for numerous physiological processes and these findings could be transferable in many cases. Furthermore, preliminary studies are often initially conducted in zebrafish before being applied to the target species to establish proof of gene function, or concept, since gene editing protocols for zebrafish are much further established. Unfortunately, there is an overall lack of instances where gene editing technologies have been applied to shellfish or crustaceans. Several difficulties further complicate the delivery of CRISPR/Cas9 into invertebrate embryos. First, bacterial and fungal infections commonly occur when culturing invertebrate organisms in vitro and have been shown to result in embryo death.[95,96] Second, the egg membrane hardens quickly after oviposition due to the presence of peroxidase and dual oxidases, thereby making it more difficult for needle insertion.[97] There is also a risk of leakage from egg components during microinjection from the difference in osmotic pressure between the inside and outside of the embryo.[98,99] Lastly, the long-term culture of many invertebrates is challenging due to their complex life histories. Despite these difficulties, gene editing has been achieved in some crustaceans such as *Exopalaemon carinicauda*,[100] *Parhyale hawaiensis*[101] and *Daphnia magna*,[102] with many of these studies focusing on producing evident phenotypes, such as eyeless mutants.[101–104]

Excluding model species, a large number of the studies reviewed were found to focus on genes related to reproduction and development (43.3%). The second greatest focus was related to pigmentation (28.3%), followed by growth (23.3%) and disease resistance (5.0%; Figure 2). Across all targeted traits, the most commonly used gene editing technology was CRISPR, followed by TALEN, and ZFN. Strikingly, the only gene editing technology associated with targeting disease resistance was CRISPR (Figure 2). This is likely due to the fact that the application of genetic technologies into the study of disease resistance within aquaculture is still a relatively new science; therefore, around the time this idea became popular, CRISPR was already established as the preferred gene editing technology[48] (Figure 3).

The development of gene editing technologies over the years has coincided with an increase in the number of topics covered per targeted trait (Figures 3 and 5). As gene editing technologies developed, their application has become more accessible, allowing for the expansion of research into lesser-known topics and species. For example, early studies often focused on zebrafish (*Danio rerio*)[105–107] and medaka (*Oryzias latipes*),[108–110] with recent studies expanding to incorporate lesser studied species, such as rainbow trout (*Oncorhynchus mykiss*)[111] and blunt snout bream (*Megalobrama amblycephala*).[112] Preliminary studies confirmed previous knowledge and gave way to the expansion of new ideas; furthermore, the development of CRISPR allowed for the targeting of multiple genes at one time.

Studies related to reproduction and development had a broader focus and utilised TALEN more than studies targeting other traits (Figures 2 and 5a). Since it is easy to validate methodology when targeting a strikingly evident phenotype, pigmentation genes are often targeted when utilising a gene editing technology for the first time or applying the technique to a new species. Considering the melanogenesis pathway is well described, it is no surprise that this pathway represented the majority of studies targeting pigment-related traits.

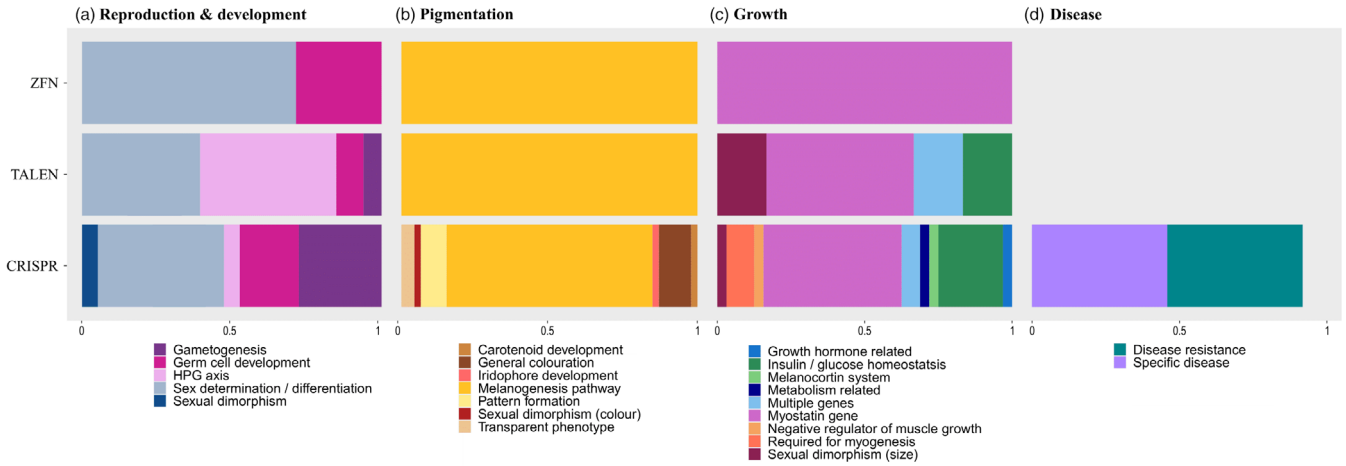


**FIGURE 5** The research focuses on the top targeted traits relevant to gene editing in aquaculture species: (a) reproduction and development, (b) pigmentation, (c) growth and (d) disease. The *y*-axis represents the type of gene editing technology used while the *x*-axis represents the focus of each study for each targeted trait and is represented as a percentage.

However, in recent years, this focus has expanded to account for other types of pigmentation cells and pathways (Figure 5b). Similarly, a large percentage of the studies targeting growth were focused on a single gene, the myostatin gene. As technology developed, the extent of research also expanded (Figure 5c). Around half of the studies targeting genes related to disease resistance focused on physiological processes relating to a specific disease, while the other half focused on general innate immunity (Figure 5d).

## 4 | TRAITS TO OPTIMISE

### 4.1 | Reproduction and development

Selective breeding programs often seek to improve reproductive and developmental traits that are valuable in aquaculture production.[113] However, there are many complexities within these traits that lead to a wide variety of phenotypes; such differences can often be attributed to differences in reproductive strategies such as sequential hermaphroditism, simultaneous hermaphroditism,[114] sexual dimorphism,[115–117] as well as gonochorism.[118–121] Furthermore, the variety of reproductive strategies leads to further complications in terms of creating a general protocol for selective breeding programs. There remains a need to further identify which genes play significant roles in the reproduction and development of aquaculture species, as well as develop a general approach to incorporate genetic techniques into selective breeding programs on a broad scale.[9]

Several studies have utilised gene editing technologies to identify functional genes that influence the neuroendocrine and endocrine systems regulating reproduction, also known as the hypothalamic–pituitary–gonadal axis (HPG). The hypothalamus functions as a master-regulator since it releases hormones and electrical signals to direct the activity of other glands.[122] This regulation usually takes the form of stimulatory and inhibitory neurohormones. Specifically, in the case of reproduction and development, the hypothalamus is responsible for releasing gonadotropin-releasing hormones (GnRH), thereby influencing various stages of reproduction. GnRH directly influences the levels of both the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH).[123] The function of GnRH has been thoroughly studied in several vertebrates; however, a limited number of studies have utilised gene editing techniques to clarify the function of GnRH in fish.[124,125] The first two studies, both utilising TALEN technology, found contradicting results, with Spicer et al. finding no influence of GnRH knockout on sexual maturation and gametogenesis in zebrafish (*Danio rerio*) due to possible compensatory mechanisms,[126] and Takahashi et al. finding the knockout of GnRH to lead to female infertility in medaka (*Oryzias latipes*).[127] More recently, Feng et al. found GnRH to regulate the proliferation of primary germ cells, thereby playing an essential role in early sex differentiation in zebrafish.[128] A subsequent study found the knockout of GnRH to induce low fertility in channel catfish (*Ictalurus punctatus*).[129] Other studies have focused on the knockout of genes related to the different factors that may regulate GnRH, such as kisspeptin protein levels (*Kiss*) and its receptor *Gpr54*.[130] One study conducted by Tang et al. knocked out genes related to *Kiss* in zebrafish; however, no change in phenotype was observed and fertility remained intact for both sexes of fish.[131] Subsequent studies targeting genes related to *Kiss* in zebrafish revealed varying levels of expression for genes related to the *Kiss* system; therefore, it was concluded that a compensatory mechanism may exist where multiple factors, such as *Kiss* paralogs, play a role with GnRH to stimulate the reproductive axis.[132–134] Therefore, it is likely that the knockout of GnRH and its precursors will not provide consistent and reliable results. With this realisation, several studies have taken a different approach.

LH and FSH are two of the master regulating gonadotrophic hormones, are part of the glycoprotein hormone family, and are released from the pituitary gland. LH and FSH are responsible for maturation, gametogenesis and steroidogenesis.[135] LH is responsible for stimulating the production of androgen and triggering oocyte

maturation and ovulation. FSH is responsible for stimulating follicular growth and oestrogen, as well as promoting spermatogenesis in the testis.[136] Recently, scientists have been able to better determine the importance of these hormones within fish models; however, only a handful of studies have attempted to elucidate the function of LH and FSH through gene knockout. A study focusing on FSH knockout in medaka (*Oryzias latipes*) found that the release of FSH does not necessarily require activation from GnRH, suggesting that the targeting of GnRH alone may not be sufficient to produce adequate effects.[127] In comparison, knockout of genes related to LH did not affect follicle growth; however, knockout resulted in failed ovulation and oocyte maturation.[127,136,137] One study conducted in zebrafish by Li et al. (2015) suggested that reduced *Igf3* expression related to the knockout of LH may actually be responsible for the anovulatory phenotype.[138] Subsequently, the knockout of *Igf3* in Nile tilapia (*Oreochromis niloticus*) resulted in the inhibition of spermatogonial proliferation and differentiation, thereby preventing spermatogenesis and reproduction.[139] Loss of genes related to FSH has been shown to result in severely delayed puberty onset and follicle activation.[127,136,140] These findings have been derived from a limited number of studies and should be further expanded for testing within a larger spread of aquaculture species.

Reproductive control in aquaculture can provide numerous commercial benefits. For example, the production of a monosex culture can give way to uniform body size, rapid growth, and a lack of spawning.[141,142] Methods, such as sex reversal, can allow for the direct pairing of superior performing individuals and enable efficient genetic gain.[143] In species that are sequential hermaphrodites; sex reversal would allow for breeding at a younger age, thereby reducing the amount of time needed to see genetic gain. For example, barramundi (*Lates calcarifer*) is a sequential protandrous hermaphroditic species that performs mass spawning. However, in Australian aquaculture, an extensive holding time is required before the fish changes from male to female, approximately 4–6 years. This reproductive strategy does not allow for the breeding of males and females of the same generation and age, thereby extending the generation interval and limiting the rate of genetic progress that can be achieved.[144] Sex reversal, thus, would decrease generation interval and increase the rate of annualised genetic gain.

Sexual development is shaped by a variety of components. Sex determination regulates the development of primordial germ cells (PGCs) into testis or ovary; alternatively, sexual differentiation occurs after sex determination. Sex determination is usually accomplished through an individual's genetic makeup or environmental influences,[145–147] while sexual differentiation is the process of testicular or ovarian development from the undifferentiated gonad.[148] A wide range of transcription factor interactions, signalling molecules and regulators constitute the genetic makeup that ultimately determines an organism's sex.[149] The production of gametes, or gametogenesis, occurs when germ cells undergo meiosis to produce haploid cells. This process takes place within the gonads, specifically the ovary (oogenesis) or testis (spermatogenesis).[150]

Recently, studies have focused on better determining which genes have an influence on gametogenesis.[151–153] Several influential genes regulating sex include, but are not limited to, double sex and mab-3 related transcription factor 1 (*Dmrt1*),[154] forkhead box transcription factor L2 (*Foxl2*)[155] and R-spondin-1 (*Rspo1*).[156] For example, the knockout of either *Foxl2* or *Cyp19a1a* results in female-to-male sex reversal in Nile tilapia (*Oreochromis niloticus*).[157] Alternatively, using CRISPR/Cas9 to knockout *Cyp19a1a* in the rice field eel (*Monopterus albus*) resulted in an arrest of ovarian development, while the knockout of *Foxl2* did not directly affect ovarian development.[158] This highlights that the knockout of the same genes in one species may not have the same impact on another species, especially in cases where there are other complicating factors, such as hermaphrodism as seen in rice field eel. Gonadal differentiation is regulated by a number of influential genes such as anti-Müllerian hormone (*Amh*),[159] anti-Müllerian hormone receptors (*Amhr2*),[160] oestrogen receptors[161] and androgen receptors.[162] When CRISPR/Cas9 was used to knockout *Amhr2* in Japanese flounder (*Paralichthys olivaceus*), male-to-female sex reversal was observed.[163] When targeting oestrogen receptors, it is important to account for all the nuclear oestrogen receptors (nERs) as well as their subtypes. Lu et al. knocked out all three nERs separately as well as double and triple knockouts using CRISPR/Cas9 in zebrafish. All single knockouts resulted in normal development, which suggests functional redundancy among the nERs. Yet, the double knockout of two of the nERs, namely *Esr2a* and *Esr2b*, resulted in the arrest of folliculogenesis and subsequent sex reversal from female to male.[164] Despite the fact that the majority of gene editing studies in aquaculture species have focused on traits related to reproduction and development, there remains a large gap in the literature for the incorporation of gene editing to induce sex determination and sexual differentiation, especially for understudied species (Figure 4). The application of gene editing for traits related to reproduction and development within the aquaculture industry would be a valuable asset since this achievement would improve overall efficacy of trait selection, as well as prevent the negative connotations associated with the use of steroidal sex hormones in the food sector.

Sterility of aquaculture species remains a hot topic. The recent popularity of inducing sterility stems from the desire to minimise rates of introgressive hybridisation of any escapees from culture systems with wild stocks. Furthermore, inducing sterility at a young age avoids the negative impacts associated with early maturation and allows the organism to focus its energy on growth.[165] Sterility may promote the development of several commercially advantageous traits such as improved flesh quality, increased growth rate, increased environmental tolerance, and increased disease resistance.[166–168] In the past, the application of surgery, irradiation, chemical and hormonal treatments have been used to induce sterility in fish.[169] More recently, triploidisation has been made a popular method for the creation of infertile species.[169–171] However, producing triploid organisms has been shown to result in a higher intolerance to suboptimal rearing conditions, as well as a higher susceptibility to deformities.[165]

The use of gene editing technologies will likely provide a more efficient and humane method to induce sterility within aquatic species. Knocking out genes responsible for germ cell formation and maintenance is one way to create a sterile line of fish.[172] In addition to improved flesh quality within gene-edited, sterile Atlantic salmon

(*Salmo salar*), these salmon displayed no adverse physiological effects.[173] The knockout of genes such as *Nanos2* and *Nanos3* have shown potential to influence germ cell development and, thus, sterility in Nile tilapia (*Oreochromis niloticus*).[174] However, the dead-end gene, *Dnd*, has been shown to be a requirement for germ cell development and shows the most potential for species-wide application.[172] Gene editing targeting *Dnd* has been applied to introduce sterility in several aquaculture species including Atlantic salmon (*Salmo salar*),[175,176] medaka (*Oryzias latipes*),[110] catfish (*Ictalurus punctatus*)[53] and zebrafish (*Danio rerio*).[177] In particular, the knockout of *Dnd* has been of interest to the salmon industry in attempts to prevent escapees from interbreeding with the genetic composition of wild stock.[178] Once technology has been further established, the knockout of multiple genes, especially including genes relating to sterility, may be recommended to maximise genetic improvement. This concept will help prevent edited alleles from being transferred to wild populations through escapees.[175,176] Gene editing targeting multiple genes has already been accomplished in several species, opening up possibilities for knocking out multiple genes in one organism. For example, using CRISPR/Cas9 in Atlantic salmon (*Salmo salar*), one group simultaneously knocked out *Dnd* to induce sterility as well as *Alb* to generate albinism.[175] However, inducing sterility on a large scale within the aquaculture industry is a difficult prospect since sterility cannot be inherited since fish lacking the *Dnd* gene do not develop germ cells. To address this issue, Güralp et al. co-injected both the wild-type variant of the salmon (*Salmo salar*) *Dnd* mRNA along with the CRISPR/Cas9 constructs, thereby performing a rescue of germ cells.[176] Therefore, the fish that were co-injected were fertile and produced germ cells, but their progeny were infertile. This represents a possible solution to generating large-scale production of sterile offspring and is highly advantageous since it does not require sex reversal or germ cell transplantation.

## 4.2 | Pigmentation

Pigmentation plays an essential role in the behaviour and life history of fishes. Differences in body colouration can affect signalling, species recognition and physiological processes.[179] Therefore, body colouration has a direct impact on mate choice, species recognition, predation, as well as protecting against ultraviolet radiation.[180,181] Body colouration sometimes changes as an organism develops and is not always permanent. Colouration is influenced by a combination of genetic and environmental factors such as light intensity, developmental stages, stress and diet.[182–184] Furthermore, handling stress from processing as well as post-processing storage can affect body and flesh colouration.[185] Pigmentation affects not only external body colouration, but also the colour of the flesh, which has direct implications on consumer bias. Previous studies surveying consumer preference suggest that participants have a much lower willingness to pay for a fish product that does not have the same colouration as those found caught in the wild.[185–188] To address this issue, farmers often incorporate dietary additives such as carotenoids, tocopherol, and ascorbic acid to influence fish colouration.[189] Furthermore, numerous breeding programs have been implemented to enhance pigment selection for body colouration.[190–192] The application of bioinformatics and genome resources have furthered the progress of breeding programs.[193,194] However, even with all of these strategies available, achieving specific body colouration is not always easy. Body colouration is often regulated by more than one gene (i.e., is a quantitative gene trait)[195] and is not always conserved through generations[196]; therefore, targeting a specific, rare phenotypic trait is relatively complex, especially if the genetic basis behind the trait is unknown.

Chromatophores, typically found within the epidermis, dermis and around the neurovascular system of teleosts, contain pigment and light-reflecting platelets responsible for body colouration.[197,198] Pigmentation cells are dispersed throughout superimposed layers, and general interactions between neighbouring segments regulate the proliferation of certain cells.[199] Chromatophores are derived from the neural crest through latent progenitors in the skin or peripheral nervous system.[200–203] There are currently 6 types of pigment cells described in teleosts: melanophores, xanthophores, erythrophores, iridophores, cyanophores and leucophores.[198] Each type of pigment cell is regulated through specific ligand-receptor pairs and cell-type-specific signalling systems.[199,204] Melanophores are chromatophores containing melanosomes that synthesise and store melanin pigments, which are responsible for black/grey colouration in fishes. When the melanin granules are dispersed, the body colour appears darker; accordingly, when the granules are concentrated towards the middle of the cells, the body appears lighter.[205] Chromatophores containing yellow pteridine pigments are described as xanthophores, while erythrophores have red/orange carotenoids.[206] However, the distinction between xanthophores and erythrophores is not always clear since vesicles are capable of containing both pteridines and carotenoids; in this case, the pigment ratio determines the colour.[207] The production pathways of xanthophores and erythrophores differ such that pteridines are generated from guanosine triphosphate (GTP) while the colouration derived from xanthophores is produced through separate biochemical pathways.[208] Guanine crystals produce the iridescence and structural colours that iridophores are known for. Cyanophores produce blue colouration; however, their pigment has yet to be discovered.[209] Lastly, leucophores contain uric acids, and scatter light producing a white colour.[181,195,208]

Since melanin pigments are most common in vertebrates, the melanogenesis pathway has been the most thoroughly described[210–213] (Figure 5b). Genes related to the production of melanin can be found throughout the genome and control a wide range of processes such as biosynthesis, differentiation, proliferation and migration.[214] One of the key genes involved in the formation of melanophore and retinal pigment epithelium is the *Mitf* gene. The *Mitf* gene, a microphthalmia-associated transcription factor, is integral to melanophore and retinal pigment epithelium (RPE) formation and acts as a 'master regulator'.[215,216] There are two paralogs of the *Mitf* gene, *Mitfa* and *Mitfb*, commonly found in teleosts. While *Mitfa* usually regulates the development of skin pigment cells, *Mitfb* mostly regulates eye and olfactory bulb development.[217] Targeting *Mitfa* through gene knockout in zebrafish (*Danio rerio*) using CRISPR did not produce a discernible phenotype.[218] However, the knockout of *Mitfa*/*Mitfb* in tilapia (*Oreochromis niloticus*) resulted in a loss of

pigmentation in the eyes, and a reduced number of melanophores.[208] Wang et al. also demonstrated the importance of knocking out both *Mitf* genes with the knockout of *Mitfa/Mitfb* resulting in dramatically increased hypopigmentation in tilapia (*Oreochromis niloticus*) compared to the singular knockout of *Mitfa* or *Mitfb*.[219] *Mitf* is also responsible for regulating several other genes affecting body colouration such as the tyrosine gene family and the melanocortin system. Melanin is produced through the synthesis of tyrosine, which is regulated through the tyrosinase gene family (*Tyr*, *Tyrp1*, *Tyrp2/Dct*, *Kit*, *Kitlga*).[220] The knockout of tyrosine (*Tyr*) has disrupted melanophore formation and altered pigmentation in numerous fishes including common carp (*Cyprinus carpio*),[221] zebrafish (*Danio rerio*),[218] tilapia (*Oreochromis niloticus*),[208] fathead minnow (*Pimephales promelas*)[222] and large-scale loach (*Paramisgurnus dabryanus*).[223] The knockout of *Tyr* through the use of CRISPR/Cas9 in zebrafish resulted in high mutagenesis rates (93%–97% within six randomly selected embryos) and an evident loss of pigmentation.[218] In the large-scale loach, knockout of *Tyr* using CRISPR/Cas9 produced an albino phenotype, with hypopigmentation in the F0 generation and complete albinism in the F2 homozygous mutants.[223] Wang et al. were also able to generate an albino mutant through the knockout of *Tyr* using CRISPR/Cas9 in Nile tilapia.[208]

The melanocortin system also plays a determinate role in skin colour and pattern[183] and is made up of numerous receptors and agonists.[184] Some of the more influential genes of this family include the melanocortin 1 receptor, *Mc1r* and the Agouti-signalling protein, *Asip1*.[224,225] The melanocortin 1 receptor, *Mc1r*, is an essential receptor in vertebrate pigmentation. In fishes, *Mc1r*, regulates the dispersion of melanosomes and is involved in the adaptation to light/dark.[226,227] In mammals, it has been shown that when *Asip1* binds to *Mc1r*, the ratio of produced eumelanin (dark pigment) to pheomelanin (yellow/red pigment) is lowered, and melanoblast proliferation and differentiation is inhibited.[228,229] Signalling from *Asip1* also plays an essential role in the distribution of dark melanophores involved in countershading.[230,231] The knockout of *Asip1* in zebrafish using CRISPR disrupted the dorso-ventral pigment pattern associated with countershading.[230] Another well-known gene essential for pigmentation, the golden gene (*Gol* or *Slc24a5*), is highly conserved across vertebrates and is involved in the synthesis pathway of melanosomes. This gene has been shown to have an influence on the number, size and distribution of melanosomes.[232] Several studies have altered pigmentation through the knockout of *Slc24a5* in fishes such as zebrafish,[233] and mackerel tuna.[234] Using TALEN, *Slc24a5* was knocked out in mackerel tuna (*Euthynnus affinis*) and resulted in reduced melanin pigments at both the early and later stages of life compared to the control.[234] Similarly, the knockout of *Slc24a5* using CRISPR/Cas9 resulted in the absence of melanophores in larvae and adult zebrafish.[233] Another popular gene concerning melanin biosynthesis is solute carrier family 45 member 2, *Slc45a2*. Microinjection of *Slc45a2* using the CRISPR/Cas9 system resulted in albinism in Nile tilapia (*Oreochromis niloticus*).[235] Zhang et al. knocked out the *Slc45a2* gene in two species of ornamental fishes, royal farlowella (*Sturisoma panamense*) and redhead cichlids (*Vieja melanura*), resulting in albino pigmentation.[236] The knockout of *Tyr* and *Slc45a2* was achieved in Atlantic salmon (*Salmo salar*) resulting in some pigment loss in the F0 generation.[237] Once the role of a gene for a particular phenotype has been confirmed, this information can help inform future projects. Once *Slc45a2* was confirmed to result in hypopigmentation in Atlantic salmon (*Salmo salar*), it was later used in a double-knockout experiment targeting both *Slc45a2* and *Dnd*; therefore, it was established that loss of pigmentation within the double-injected embryos indicated there might be a high mutation rate for the second targeted gene.[175] This double-injection technique holds to be very promising for future endeavours since hypopigmentation phenotypes are strikingly evident and recognition of this type of phenotype may require less downstream genetic work. Other genes shown to have an influence on melanin production through a combination of gene knockout, gene expression and genome-wide association studies include *Pmel*, *Hps4*, *Slc7a11* and *Gata2*.[220,238–243]

Pigmentation development within other pathways has been less studied. For the migration and differentiation of xanthophore precursors, genes such as *Gch1/Gch2*, *Csf1r* and *Pax7* have been shown to be of influence. Similarly, genes such as *Scarb1*, *Bco*, *Gch1/Gch2*, *Spr*, *Pts* and *Xdh*, play a role in the metabolism of xanthophores and erythrophores.[181,244–247] Pteridine is synthesised from GTP, which is regulated by several enzyme genes such as *Gch1/Gch2*, *Pts* and *Spr*.[211,244,245] Alternatively, carotenoids are not produced in teleosts, but this pigment can be accumulated and processed from their diet. Iridophores play a significant role in the formation of stripes in zebrafish.[204] A combination of gene knockout and cell transplantation studies have shown that genes *Pnp4a* and *Mpv17* influence the production of guanine.[248–251] These cells only proliferate and spread once they reach the skin. In addition, endothelin genes, such as *Edn3a* and *Edn3b*, may potentially influence the presence of iridophores; genetic and transgenic approaches were used in zebrafish to identify *Edn3a* and *Edn3b* as genes that contribute to the reduction of iridophore proliferation.[252]

Since pigmentation gene editing is convenient to analyse through phenotypical differences, pigmentation genes are often targeted to fine-tune protocols.[78] Zebrafish have long served as a model for gene editing since they are able to give rise to a large number of progeny and have proven to be amendable to numerous forms of genetic manipulation.[253] Therefore, it is no surprise that all three major platforms of gene editing have been utilised for targeting pigmentation genes in zebrafish (Figures 3 and 5b) and have resulted in phenotypic alterations as well as the transmission of mutations and phenotypic traits to progeny.

## 4.3 | Growth

Growth rate is another commonly targeted trait for gene editing within aquaculture species.[85] For fishes, major growth is achieved by skeletal muscle accretion through the process of cellular hypertrophy and hyperplasia.[254] The processes involved within hypertrophy and hyperplasia differ. The increase in diameter of muscle fibre, or hypertrophy, occurs when the size of muscle fibres are increased.[255]

Alternatively, an increase in the number of muscle fibres is known as hyperplasia.[256] Overall growth can be influenced by hormones and myogenic regulatory factors (MRFs). Essential hormones within the endocrine system, such as growth hormones and insulin-like growth factors, are able to modulate the growth and development of muscle tissues.[257] However, hormones regulate other essential physiological factors besides just muscle development. Hormones such as prolactin, somatolactin and growth hormone play other additional roles such as the development of bladder inflation, head and eye size, as well as the adjustment to changes in salinity.[258] Since the overexpression of growth hormone has been shown to result in decreased myostatin transcript and protein expression, targeting MRFs is a straightforward way to influence muscle development without pleiotropic effects.[259]

Muscle development through MRFs is primarily accomplished through two different protein synthesis pathways: the mTOR pathway, which acts as a positive regulator, and the myostatin-Smad2/3 pathway, which acts as a negative regulator. However, the mTOR pathway is essential for several other crucial cellular processes such as autophagy.[260] The myostatin pathway is part of the transforming growth factor-β (TGF-β) superfamily. Several cellular growth and differentiation processes are regulated by the TGF-β superfamily.[261] Specifically, growth/differentiation factor-8, now known as myostatin (*Mstn*), negatively regulates muscle fibre growth through the inhibition of myoblast proliferation and differentiation.[262–265] Therefore, when the myostatin gene is inactivated or knocked out, a decrease in *Mstn* expression and an increased rate of growth/number of muscle fibres are revealed.[262,266] The amino acid precursor protein of this gene is composed of a signal sequence, an N-terminal propeptide domain and a C-terminal domain with an active ligand. The mature form of the myostatin gene is achieved through two steps of proteolytic processing between the propeptide and C-terminal domain.[262,267] Successful inhibition of *Mstn* activity has been achieved via N-terminal propeptide overexpression, morpholino or double-strand RNA interference.[268–270] Accordingly, the myostatin pathway is often targeted in LOF studies since gene knockout has been shown to result in increased muscle growth[106] (Figure 5c).

The first described mutations of the *Mstn* gene were reported in cattle breeds, namely Belgian Blue and Piedmontese, with these breeds displaying a noticeably higher muscle mass compared to conventional cattle.[271,272] Findings of natural mutations in the *Mstn* gene sparked interest to extend this study to other breeds and species. The *Mstn* gene has been cloned and characterised for numerous commercial fish species of high value.[273–285] However, a lack of polymorphism within the coding regions of these fish suggests that natural mutations may be less likely to occur in fishes compared to higher vertebrates.[267,286,287] Throughout evolution, the *Mstn* gene has remained well conserved among fishes and vertebrates as a whole.[282,288] The knockout of myostatin in mice has led to an increase in hyperplasia and hypertrophy resulting in an increase in skeletal muscle mass.[267] In addition, some studies have shown reduced fat content when *Mstn* was inactivated and increased fat content when *Mstn* was overexpressed,[289,290] indicating a direct correlation between fat content and *Mstn* expression. Inhibition of the *Mstn* gene in fishes has been shown to result in an increased quantity of myofibers without significantly affecting the size of muscle fibres.[270]

Generally, myostatin is expressed in developing skeletal muscle as well as the heart of higher vertebrates.[267,291] Alternatively, piscine myostatin has been detected in various other tissues including gill filaments, eyes, ovaries, brain, gut, spleen and testis,[276,277] and isoforms of the *Mstn* gene have been isolated from a variety of species.[273,275,292,293] As previously highlighted, unlike higher vertebrates, fishes may have more than one copy of *Mstn* within their genome, most commonly *Mstn*-1 and *Mstn*-2. While *Mstn*-1 generally displays a continuous expression pattern, *Mstn*-2 was found to be associated with somitogenesis. Furthermore, *Mstn*-2 is not always found within all fish lineages.[294–296] This further emphasises the importance of considering ancestral whole-genome duplication when designing targets for gene editing.[93] Recently, there have been numerous successful examples of *Mstn* gene knockout leading to the promotion of muscle growth in aquaculture species[112,297–301] (Figure 5c). The knockout of *Mstn* in common carp (*Cyprinus carpio*) through the use of CRISPR/Cas9 resulted in a 51.4% increase in skeletal muscle fibre density and a significantly greater body size compared to the wild-type; in addition, there were no off-target effects or signs of visible, physical deformity.[297] When the *Mstn* gene was knocked out with CRISPR/Cas9 in channel catfish (*Ictalurus punctatus*), the mean body weight of the gene-edited fry were 29.7% greater compared to the control.[302] Similarly, the knockout of *Mstn* through CRISPR/Cas9 in blunt snout bream (*Megalobrama amblycephala*) also resulted in a significant increase in the body height, weight and overall thickness of *Mstn* deficient fish compared to the wild-type control.[112] Taking the results from these studies into consideration, it is no surprise that the *Mstn* gene is most commonly targeted to influence growth (Figure 5c).

Several other studies have attempted to influence muscle growth without directly knocking out the *Mstn* gene. The activin A receptor, type II (*Acvr2*), is also a member of the TGF-β superfamily. Like *Mstn*, *Acvr2* negatively regulates skeletal muscle mass.[303] Using a knockout of *Acvr2*, Che et al. showed an increase in muscle hypertrophy in zebrafish (*Danio rerio*).[304] Stress is also capable of influencing several metabolic, physiological, and behavioural processes through the actions of corticosteroids and catecholamines. In addition to playing a role in stress, the melanocortinergic neural circuit is also known to influence feeding behaviour and energy expenditure.[305] Melanocortin receptors (MCRs) function as transmembrane proteins within the G-protein-coupled receptors superfamily. Specifically, *Mc4r*, has been found to play an integral role in energy homeostasis and the regulation of food intake.[306,307] Recently, Khalil et al. knocked out *Mc4r* in channel catfish (*Ictalurus punctatus*) using CRISPR/Cas9. This knockout resulted in a 38% and 20% improvement in body weight for electroporation-mediated and microinjection gene editing, respectively. In addition, the average feed conversion ratio of the edited fish was 1.18 compared to 1.57 in the control fish.[308] If applicable to other species of fishes, the results from this study could greatly benefit aquaculture production.

Moreover, one of the key regulators of the melanocortin system is the pro-opiomelanocortin precursor peptide or *Pomc*. The absence of some of the biologically active components produced by *Pomc* has resulted in body weight gain in humans and mice.[309,310] However, unlike other vertebrates, the knockout of *Pomc* does not result in obesity. The knockout of *Pomc* in zebrafish resulted in the production of

phenotypes related to stress response and a gain in body weight, without obesity.[311] The knockout of *Pomc* has been particularly effective in sexually dimorphic fish.[312] Sexual dimorphism can result in significant anatomical differences between male and female individuals, such as differences in growth rate, body size, shape and colour.[116,117] This study led to the realisation that higher oestrogen levels in females were promoting the expression of *Pomc*, thereby suppressing the feeding of female zebrafish.[312] Similarly, the knockout of *Stat5.1* in zebrafish showed an attenuation of sexual size dimorphism, although with a reduction in body length and weight.[313] Other studies have focused on knocking out certain genes to influence metabolism and energy homeostasis, thereby increasing somatic growth and feed conversion efficiency.[314–316] For example, the use of CRISPR/Cas9 to knockout leptin receptor (LepR) genes, *Lepra1* and *Lepra2*, in rainbow trout (*Oncorhynchus mykiss*) resulted in a faster growth rate, greater energy mobilisation and a heavier body weight.[314] Another example is the knockout of the glucocorticoid receptor in zebrafish using CRISPR/Cas9; this resulted in a higher body mass, as well as increased protein and lipid content.[316]

## 4.4 | Disease resistance

The incorporation of genomic screening and selection into selective breeding programs allows for the identification of genes involved in disease resistance and potential strategies for rapid improvement.[317] Although some species, such as Atlantic salmon (*Salmo salar*),[318–320] channel catfish (*Ictalurus punctatus*)[321,322] and rainbow trout (*Oncorhynchus mykiss*)[323,324] have been bred for the enhancement of disease resistance, most fish production is based on stocks without a selective breeding program, despite the fact that disease resistance is predominately heritable.[325–327] Establishing selective breeding programs for disease resistance can be quite challenging, especially with the high number of effective diseases and lack of availability of proven disease resistance measures.[328] Disease resistance is usually assessed through pathogen challenges involving pedigreed populations, allowing for the quantification of disease-resistant traits through the measurement of survival and pathogen burden.[325,326,329] Other measures include using cell culture to measure pathogen or parasite load,[330] as well as examining biomarkers of host immune response.[326] Genetic information from QTL or marker-assisted selection can also provide valuable insight as to which genes may influence disease resistance[331–333]; however, it is also important to note that finding a QTL with a very large effect on a disease resistance trait is not the norm.

Only recently, studies have focused on the use of gene editing to facilitate the development of enhanced resistance to disease within broodstock.[334–336] Attempting to edit genes to promote disease resistance remains a new science. More recently, gene editing studies have primarily focused on elucidating gene function and identifying genes related to disease resistance and susceptibility. The immune and endocrine systems are primarily responsible for initiating a response towards stress or disease.[337] Therefore, gene editing research for disease-resistant traits has primarily focused on innate immunity or gene function after exposure. Studies focusing on innate immunity have targeted immune-related genes that regulate gene expression or disturb certain pathways.[338–340] Pattern recognition receptors (PRRs) detect the presence of pathogen-associated molecular patterns.[341,342] In fishes, retinoic acid-inducible gene receptors (RIGR) and toll-like receptors (TLRs) are two key classes of PRRs that play a major role in innate immunity.[343–347] TLRs and RIGR are membrane glycoproteins that activate interferons or nuclear factor pathways once they recognise pathogen-associated molecular patterns on the surface of a cell.[345,348–351] Several recent studies have focused on knocking out genes related to TLRs to better characterise their role in innate immunity; these studies included rohu carp (*Labeo rohita*), zebrafish (*Danio rerio*) and olive flounder (*Paralichthys olivaceus*).[338,339,352] Additional studies have targeted genes in fishes that are known to affect immunity in mammal models allowing for a better understanding of the immune pathways present in fishes.[353,354] For example, Ouyang et al. knocked out *Mavs*, a gene known for antiviral innate immunity in mammals, using CRISPR/Cas9 in zebrafish. In the *Mavs* deficient fish, a reduction of inflammatory and antiviral-responsive genes was evident; thus, when *Mavs* was overexpressed, cellular antiviral responses were enhanced.[355] Interestingly, Chen et al. demonstrated that *Sirt5*, a member of the sirtuin family proteins, was responsible for negatively regulating *Mavs*. When *Sirt5* was knocked out in zebrafish, genes with antiviral response were enhanced, replication of spring viremia of carp virus (SVCV) was diminished, and survival rate was increased after the fish were challenged with SVCV. In certain cases, some genes are responsible for suppressing cellular antiviral responses; for example, Yu et al. knocked out a single gene, *Phd3*, that led to an increased rate of survival when zebrafish were exposed to SVCV.[340] Findings such as these have the potential to have a significant impact on the future of breeding programs and disease response in aquaculture.

Identifying genes responsible for major QTL will greatly support selective breeding programs and streamline genetic improvement. Rarely does one single locus account for all of the genetic variation related to the resistance for a specific infectious disease. When studying infectious pancreatic necrosis virus (IPNV) in Atlantic salmon (*Salmo salar*), Pavelin et al. found the causative gene responsible for the major QTL in regard to IPNV resistance.[356] Therefore, when the causative gene was knocked out using CRISPR/Cas9, there was a significant reduction in IPNV replication. Another prominent disease in aquaculture is grass carp reovirus (GCRV); GCRV has been highly detrimental to the grass carp industry, resulting in huge economic losses.[357,358] It is difficult to treat GCRV infection since there are multiple genotypes of GCRV.[359] One study conducted by He et al. focusing on rare minnow (*Gobiocypris rarus*) found the knockout of a gene responsible for playing an important role in virus-induced apoptosis, *Bid*, resulted in delayed GCRV replication and lessened associated GCRV apoptosis.[334] Another study conducted by Ma et al. found the knockout of the *JAMA* gene to result in reduced GCRV infection in grass carp (*Ctenopharyngodon idellus*) kidney cells.[360] These studies highlight that multiple genes can influence disease resistance. Viral nervous necrosis (VNN) disease caused by nervous necrosis virus (NNV) is also a major disease resulting in considerable economic loss

in aquaculture. Yang et al. used gene knockout in zebrafish to target a causative gene, *Rrm1*, for NNV resistance that may be applied for genetic improvement in the future.[361]

Previously, the majority of research has utilised the CRISPR/Cas9 system; however, the variant endonuclease Cas13 can effectively target RNA.[362] Similar to Cas9, Cas13 uses guide RNA to recognise the substrate, which is RNA instead of DNA.[363] In fact, the use of CRISPR/Cas13 to disturb cellular transcripts, as well as modulate and edit the transcriptome has been validated by several research groups.[364,365] A new subtype of Cas 13 called CRISPR/Cas13d (CasRx) has been recently identified; CasRx has been shown to be more robust and efficient compared to other Cas13 effectors.[366,367] The CasRx system has previously shown strong specificity against targeted viruses in *Nicotiana benthamiana*; in fact, CasRx was capable of targeting two viruses simultaneously.[368] The feasibility of utilising CRISPR/Cas13 as an antiviral for single-stranded RNA (ssRNA) viruses has been demonstrated in mammalian cell lines against a wide variety of ssRNA viruses.[369] More recently, Wang et al. demonstrated the capability of targeting an RNA virus with CRISPR/CasRx in fish; they targeted the red-spotted grouper nervous necrosis virus (RGNNV) using CRISPR/CasRx and found the system to have a high interfering efficiency against RGNNV in grouper (*Epinephelus coioides*) spleen cells and an ability to inhibit viral RNA when the system was applied through direct intracranial injection. In this case, the CRISPR/CasRx system worked through the inhibition of virus replication.[370] This development paves the way for future applications of the CRISPR/CasRx system targeting viral pathogens in fishes and other vertebrate species.

## 5 | GENE EDITING IN CELL LINES

An important, intermediate step in genome trait selection could be the incorporation of gene editing in cell lines before application in whole organisms. Preliminary testing allows for the selection of sgRNAs that have the highest targeting efficiency for a specific gene before application in the whole organism. In addition, preliminary testing may allow for the confirmation of putative causative gene(s) and enables a better understanding of cellular processes underlying growth, function, and overall health. However, it is important to note that cellular processes within cell lines may not always completely resemble what is physiologically occurring at the organismal level. Preliminary testing in cell lines in place of whole organisms is cost-efficient, offers faster results, and eliminates the constraint of limited fish embryos.[371] Recently, the development of piscine cell lines has increased globally; lines have been generated from numerous types of fish tissue such as muscle, gill, liver and kidney.[372]

Piscine cell lines have further facilitated the advancement of medical research by serving as a model for certain physiological processes involved in human disease, such as genetic regulation, carcinogenesis and toxicology.[81] The majority of past work on piscine cell lines has focused on immunological studies and disease diagnostics.[79,81] In terms of aquaculture, numerous piscine cell lines encompassing a wide variety of species have been tested for susceptibility upon exposure to commonly found viruses.[79,81] When searching for disease resistance traits for viral and bacterial diseases, a potentially powerful and informative technique would be gene editing within cell line models to confirm relevant genes.[373] When the exact gene is not defined, random mutations along certain target sites can be implemented to identify functional disease-resistant genes related to qualitative traits.[374] This technique may be highly beneficial when there are no prospects or remarkably evident causative genes. The development of genome-wide CRISPR knockout (GeCKO) has enabled the screening of cell cultures (e.g., a pathogen challenge) to provide multiple target genes responsible for a desired phenotype capable of responding to the challenge.[375,376] Previously, this technique was utilised to identify a norovirus receptor within a murine cell line.[377] The application of this platform could streamline the identification of genes responsible for physiological pathways involved in disease resistance and better inform selective breeding programs in commercial aquaculture. The further development of immortal piscine cell lines that remain continuously available for research would expedite the diagnostic capabilities of GeCKO and overall response to the outbreak of disease.[9,77]

Compared to gene editing technologies conducted at the embryo stage, implementing gene modification within piscine cell lines to target traits valuable to the aquaculture industry has been less explored. Initially, RNA interference was used to attempt gene knockdown in piscine cells.[378] More recently, plasmids and viral vectors have become widely used delivery strategies of CRISPR for cellular transfection and transduction.[34,379] A study conducted by Dehler et al. was the first example of gene editing using CRISPR for gene knockout in a piscine somatic cell line. First, their embryonic cell line derived from Chinook salmon (*Oncorhynchus tshawytscha*) was designed to overexpress a monomeric form of *EGFP* (green fluorescent protein). Then, they used a plasmid-based CRISPR/Cas9 system to target the *EGFP* gene for inactivation, resulting in a gene targeting efficiency of approximately 35%.[80] Several other studies performed similar plasmid-based experiments with varying rates of success.[354,360,380,381] More recently, Ma et al. knocked out a gene in permissive grass carp (*Ctenopharyngodon idellus*) kidney cells coding for immunoglobulin member, *JAMA*. This immunoglobulin member acts as a receptor for reovirus, thereby supporting reoviral infection. These results were applied to the whole organism, and the knockout of the *JAMA* gene resulted in reduced grass carp reovirus infection in two different genotypes.[360,382] In another example, Pan et al. were able to accomplish proof-of-concept gene editing in a medaka (*Oryzias latipes*) cell line.[383] For mammalian cell lines, lentivirus, retrovirus, adeno-associated virus, as well as adenovirus have been used to deliver CRISPR/Cas9 components for gene editing.[384] So far, there has only been one successful instance of utilising viral delivery of CRISPR/Cas9 components in piscine cell lines; the editing of a salmonid (*Oncorhynchus tshawytscha*) fish cell line through the use of a lentivirus delivery method was achieved with an efficiency of 90%.[91]

Another obstacle for gene editing within piscine cell lines is the selection of an appropriate promoter. For the vector-based CRISPR/Cas9 system, an RNA polymerase (Pol III) promoter like H1 or U6 is typically used for gRNA production, while a Pol II promoter is used for

Cas9 expression; however, the large size of the complete CRISPR/Cas9 system can present some difficulties, especially when introduced into a viral vector.[69,70,385–387] Several universal promoters that have been used across diverse vertebrate taxa, such as SV40 (simian vacuolating virus 40), CMV (cytomegalovirus) and CAG (hybrid promoter consisting of CMV enhancer, chicken beta-Actin promoter and rabbit beta-Globin intron), have resulted in effective expression in fish cells from species such as the swordtail platyfish (*Xiphophorus xiphidium*), Chinook salmon (*Oncorhynchus tshawytscha*), chum salmon (*Oncorhynchus keta*) and *epithelioma papulosum cyprini* (EPC, epithelial cells isolated from fathead minnow).[388–390] However, the expression strength of the heterologous promoters within fish cells can vary greatly across species[391] and cell type.[392,393] Thus far, successful gene editing has been achieved with the use of CAG in a grass carp (*Ctenopharyngodon idellus*) cell line[360] and with the use of the CMV promoter in a Chinook salmon (CHSE; *Oncorhynchus tshawytscha*) cell line.[80] Species-specific endogenous promoters, such as EF1-alpha and beta-actin, may be valuable alternatives to the traditional promoters, and may even exceed expression strength in some cells compared to standard universal promoters.[394] For example, fish beta-actin was found to have much greater expression strength over standard promotors such as CMV when tested in an *epithelial papulosum cyprinid* cell line.[395] This was also the case with the use of endogenous beta-actin promoters in a Mozambique tilapia (*Oreochromis mossambicus*) cell line.[71] However, the effectiveness of these promoters can be unpredictable; in Japanese flounder (*Paralichthys olivaceus*) cells, CMV was actually found to be stronger than endogenous beta-actin promoters.[396] Therefore, the evaluation of possible Pol II promoters should be an important step in protocol optimisation before application in downstream operations. Another option is the direct transfection of cells with gRNA/Cas9 RNP. The delivery of RNP is usually achieved through lipofection or electroporation. The advantage of RNP is the lower risk of off-target effects due to its immediate activation and more rapid rate of degradation.[397–400] Liu et al. transfected medaka (*Oryzias latipes*) fish cells with an RNP complex through electroporation and were able to achieve 50% gene editing efficiency in haploid cells with their highest mutation efficiency of 61.5% in diploid cells.[401] Zoppo et al. also utilised an RNP complex and achieved a 39% gene editing efficiency within rainbow trout (*Oncorhynchus mykiss*) cell lines.[402] More recently, Strømsnes et al. used a combination of strategies involving both RNP and plasmid CRISPR/Cas9 to edit gene loci in salmonid (*Salmo salar*) cell lines.[403] The use of RNP complexes for transfection continues to receive much attention for editing within cell lines.[404]

## 6 | THE INCORPORATION OF GENE EDITING INTO AQUACULTURE PRODUCTION

The incorporation of gene editing within the aquaculture industry has the potential to expedite genetic improvement through the rapid fixation of desirable alleles by incorporating favourable alleles from wild stock and/or introducing advantageous de novo changes that currently do not exist in nature through gene knockouts or knock-ins.[9,26,77] By introducing targeted edits into the breeding population, this eliminates years of selective breeding to fix commercially desirable alleles. At the moment, gene editing mostly remains at the research stage and implementation within aquaculture breeding programs is in its infancy. Nonetheless, several companies, such as the Kyoto-based start-up Regional Fish Institute with Kyoto University and Kindai University,[405] have shown incorporation within the aquaculture industry is a possibility.[406,407] Several considerations must be made when implementing gene editing technologies into fish production. For instance, when delivering the CRISPR/Cas construct into the fish, it is preferred to be inserted into a freshly fertilised fish egg at the one-cell stage.[408,409] The time frame of embryonic development can vary greatly from species to species. Once the time frame from fertilisation to the first cell division is realised, it is possible to inject hundreds of individuals at a time.[410] However, it can be challenging to microinject a large number of eggs within a short period of time and is, therefore, a labour-intensive process requiring specific training for microinjection. Depending on the breeding program, this may be sufficient for breeding purposes. However, further breeding may be necessary to obtain homozygous edited individuals. The incorporation of gene editing could allow for the fixation of favourable alleles and/or the elimination of harmful alleles after performing an $F_1$ cross to double favourable recessive alleles, depending on the extent of mosaicism and heterozygosity.[410,411] The confirmation of homozygote-edited offspring would require an additional step of genetic screening, such as the identification of indels through the PCR-based method of heteroduplex mobility assay or next-generation sequencing approaches.[409] Depending on the desired types of gene edits, the company may even choose to commercialise different lines with varying degrees of edits.[412]

Particular attention must be paid to the implementation and monitoring of gene editing within broodstock to ensure the greatest level of success. A risk–benefit analysis of the use of gene editing in aquaculture is also recommended before commencing.[412] For the most rapid improvement of target traits, simultaneously knocking out multiple genes within the same broodstock would be recommended.[410] This may involve splitting the breeding nucleus and overseeing both edited and non-edited individuals separately or maintaining a single breeding nucleus for edits to be made to select individuals at the multiplier level.[412] Throughout this process, it is important to monitor the status of gene editing. In this case, limiting the level of mosaicism and off-target effects would be essential. The further development of gene-editing methods and protocols will help reduce these effects and help streamline the monitoring process. It is also important to consider the maintenance of genetic variation within the edited population to avoid high levels of inbreeding.[10] Initially, there might not be that many individuals carrying the desired edits, thereby limiting the number of potential pairings. Therefore, at the beginning, it might be necessary to have an initial round of gene editing with thousands of injected individuals to increase the variation and level of successful editing. Theoretically, once a successfully edited broodstock has been

established, the desired edits should be carried throughout the population.

Although the use of the CRISPR/Cas9 system is effective in terms of studying gene function, for some species, this system is unable to generate a sufficient quantity of fish for breeding purposes due to the short time frame of embryo production and growth.[9] Therefore, in order to further accelerate genetic improvement on an industry scale, without severely affecting selection accuracy, several possibilities exist to further reduce the generation time needed to observe appreciable genetic gain in relevant cases. Although these technologies are novel, they have the potential for profound and favourable impacts on accelerating trait selection in aquaculture, especially for species with longer generation intervals and species that are challenging to rear in captivity.[9] Sperm-mediated transfer remains a possibility for expediting genetic improvement on a large scale.[413] The modification of sperm before fertilisation allows for large-scale gene transfer that would be highly beneficial for the aquaculture industry, especially in species where other gene editing approaches are not possible. However, this technology has not been popular in the past due to its low efficiency[413,414]; recent advances, such as new gene editing technologies, may allow for this technology to become more plausible in the future.[415]

Looking forward, the development of surrogate broodstock technology may be one of the more promising approaches to further advance genomic improvement on a large scale.[416] The editing of the germline within broodstock animals is a potential approach for the production of genetically edited organisms on a large scale and should be considered for future application within the aquaculture industry. However, similar to gene editing within the broodstock, it is important to consider certain limitations such as the maintenance of genetic variation. Surrogate broodstock technology involves the transplantation of germ cells into sterilised recipients.[416] Germline stem cells are isolated from PGCs or gonial cells and inserted into sterile recipients. The main limitation of harvesting PGCs is the fact that each embryo only has around 13–43 PGCs.[417] Although PGCs have been shown to be capable of growing in culture,[418] there is a general lack of knowledge for culturing PGCs, especially in understudied fish species. In comparison, gonial cells can be isolated from sexually differentiated organisms. Although the gonial cells are in a sexually differentiated state, they actually have sexual biopotency and are capable of generating sperm or eggs in regard to the phenotypic sex of the surrogate.[419–422] In comparison to PGCs, gonial cells are much more abundantly found within an organism and the in vitro culture systems are more developed, with numerous successful examples.[423–426] Once isolated, germ cells are transplanted into a sterile organism. The transplantation can take place at several different life stages such as blastula, hatchlings and adults.[416] The life stage at which transplantation takes place is dependent upon several factors. Transplantation usually takes place at the hatchling stage since this stage enables the use of gonial cells. Additionally, the immune system at the hatchling stage is less likely to reject the transplantation.[427] At the blastula stage, transplantation is impractical for large-scale production since PGC isolation is required.[428] At the adult stage, gonial cell transplantation is also achievable, allowing for production in a shorter period of time; however, the success rate of transmission is lowest at this life stage.[429] Despite a lower rate of success, transplantation at the adult stage is sometimes required, such as in cases where the depletion of PGCs results in masculinisation or for use in sequential hermaphroditic species.[430]

Numerous benefits can be derived from the incorporation of surrogate broodstock technology for gene editing in aquaculture. Surrogate broodstock technology is able to shorten the interval at which highly beneficial gene edits can be disseminated throughout a breeding program.[416] Therefore, this technology may be beneficial when there are certain alleles that may be advantageous to carry during disease outbreaks or certain seasonal variations. Another potential application of surrogate broodstock technology is the production of progeny in species that are easier to rear in captivity, or that have shorter life spans. Xenogenic transplantation has been successfully accomplished in several species.[431–433] Additionally, germ cells can be cryopreserved, allowing for the preservation of highly beneficial alleles or germ cells from endangered species.[434] Furthermore, surrogate technology is capable of addressing some of the negative consequences associated with standard gene editing such as mosaicism. Although this can be addressed with technologies that attempt to only induce double-stranded breaks at the one-cell stage,[435–437] surrogate broodstock technology can overcome this issue by ensuring that progeny will receive cells that are known to have carefully selected, targeted edits. Accordingly, this will also help prevent edited populations from carrying a variety of different edited alleles.[416]

Several important considerations must be made when incorporating surrogate broodstock technology into aquaculture production. Similar to gene edits made within the standard breeding nucleus, it is also important to consider the maintenance of genetic variation when introducing edited germline into surrogate broodstock.[10] Thus, the number of surrogate donors will be an important consideration for the maintenance of genetic diversity of the aquaculture population. Theoretically, it would be beneficial for the number of surrogate donors to mimic that of the standard breeding nucleus in order to maintain the genetic variation at the same level the farm currently operates. The dissemination of edited germline requires a sterilised recipient.[416] However, it may be considered risky to sterilise important broodstock fish. This may require a separate, secondary population of sterilised fish, which many farms do not necessarily have the capacity for this type of operation. Surrogate broodstock technology has been successfully achieved experimentally in several species,[111,438–440] and shows great promise for incorporation into the aquaculture industry. For example, gene editing has been used to knockout the *Dnd* gene in rainbow trout to produce sterilised fish for use as recipients during germ cell transplantation. After the knockout of the *Dnd* gene in rainbow trout (*Oncorhynchus mykiss*), the crossing of $F_1$ heterozygous fish resulted in around ¼ homozygous fish with *Dnd* gene knockout. At the age of 1 year, these fish were completely absent in germ cells. Subsequently, germ cell transplantation was performed on the sterile fish resulting in these individuals only producing sperm and eggs from the donor fish at a rate similar to what is seen in wild-type rainbow trout.[111] This indicates that this species of fish may be suitable for

surrogate broodstock technology. Other instances of germ cell transplantation involved species-to-species transfers. For example, since the grass puffer (*Takifugu alboplumbeus*) is able to reach maturity at a much faster rate compared to the commonly cultured tiger puffer (*Takifugu rubripes*), germ cell-deficient grass pufferfish were produced through gene knockdown and germ cells extracted from the tiger puffer were transplanted into the grass puffer.[439] A similar concept was applied in two species of carp (*Cyprinus carpio* and *Carassius auratus*)[438] as well as in salmonids (*Oncorhynchus masou* and *Oncorhynchus mykiss*).[440] This highlights the beneficial use of surrogate production in order to accelerate the breeding progress. Once the technology has been further improved and is highly replicable, this technology may replace the standard breeding strategy in certain cases. However, due to the research and development involved within this technology, it may be more beneficial for species where standard methods of gene editing are not as developed or applicable.

## 7 | PUBLIC AND REGULATORY APPROVAL OF GENE EDITING IN AQUACULTURE

Previously, gene editing has been explored within the livestock industry to improve the efficiency of animal breeding programs through the cumulation of permanent and heritable mutations.[441,442] Similarly, the incorporation of this approach has the potential to transform the aquaculture industry. However, several obstacles hinder the incorporation of biotechnological tools into selective breeding programs. The utilisation of genetic technologies within selective breeding programs is dependent upon regulatory, political, social and economic landscapes, especially within low- and middle-income countries.[443] There has been much controversy over the use of gene editing when applied to the food production sector.[444] Concerns have centred around animal ethics, risks to human health or the environment, as well as research motivation.[445]

Genetic techniques that use site-directed nucleases (SDN), such as ZFNs, TALENs and CRISPR, have been further characterised by the European Food Safety Authority (EFSA).[446] EFSA has defined three categories of SDNs: SDN-1, SDN-2 and SDN-3. Systems involving SDN-1 repair introduce a specific point mutation that results in gene disruption, gene knockout or gene silencing. SDN-2 systems aim to modify a single or small number of bases through the use of a repair template. Gene editing involving SDN-1 or SDN-2 repair is generally more accepted because these mutations are indistinguishable from natural mutations.[10,447] Despite the fact that SDN-2 mutations are also considered similar to mutations that occur naturally, they are still considered modified organisms under the Cartagena Act and are considered on a case-by-case basis in most countries.[448] Alternatively, SDN-3 systems incorporate a repair template consisting of a homologous region of 500 or more base pairs at both the upstream and downstream ends of the target region. SDN-3 is used to introduce a new sequence at the target site, commonly known as gene knock-in.[446]

Policies regarding the regulation of gene-edited organisms varies per country. Tachikawa and Matsuo defined the four main regulatory approaches that can be partitioned into two main positions regarding the manner in which countries tend to regulate gene editing.[449] The first position subjects gene-edited organisms to regulations associated with genetic modification. Accordingly, this position involves either applying already existing genetically modified organism (GMO) regulation as is or applying a more simplified regulation.[449] The European Union and the New Zealand Environmental Protection Agency both consider gene-edited organisms to be under the same regulations as GMOs. China and the United Kingdom are working towards adopting a framework that allows them to authorise the use of gene-edited organisms through a more simplified procedure, while still considering them within the overarching regulatory framework of GMOs. The second position is the exclusion of gene-edited organisms from the regulatory scope of GMOs.[449] As a result, some countries may decide to require prior confirmation from the government, such as for Argentina and Japan, while other countries allow developers to make the decision without prior confirmation from any regulatory agency (i.e., US Department of Agriculture and the Australian Office of Gene Technology).[449]

Fortunately, public perception on gene editing has improved over time.[450] In fact, the first instance of gene-edited fish being marketed to the public occurred in Japan. In 2021, two fish species that were gene-edited, red sea bream (*Pagrus major*) and tiger pufferfish (*Takifugu rubripe*), were approved for the market. Using CRISPR/Cas9, the myostatin gene (*Mstn*) was knocked out from the red sea bream and the leptin receptor gene (*Lepr*) was knocked out from the tiger pufferfish.[406,407] This resulted in red sea bream lacking a gene that suppresses muscle growth and tiger pufferfish lacking a gene that controls appetite. Altogether, these edits resulted in an increased fillet yield for both species.[451] It is important to note that the science behind these examples of gene editing was not peer-reviewed and the announcement of these gene-edited fishes was carried out through newspaper publicity; therefore, it is not entirely clear what exact methods were used to create these fishes or what kind of impact this may have for the aquaculture industry in the future. However, since these species had SDN-1 mutations that did not insert any new genetic material, the Japanese Ministry of Health, Labor and Welfare (MHLW) determined that the two gene-edited fishes were not considered modified organisms[452] According to this determination, the fishes did not have to be subjected to a requisite food safety review. They were also not subject to regulation under the Japanese Cartagena Act.[453] For further reassurance, whole-genome sequencing and PCR analyses were conducted on these species to ensure that no foreign sequences or off-target mutations were present. In addition, protein allergenicity tests were conducted.[453] Another example of an aquatic product submitted for regulatory review with an SDN-1 mutation is the *Mstn* gene knockout line of FLT-01 Nile tilapia (*Oreochromis niloticus*) developed through the AquaBounty Company.[454] This tilapia had increased fillet yield compared to its standard counterpart.[455] Similar to the fishes in Japan, the tilapia did not contain any new genetic material. Hence, the tilapia was not considered a GMO under Argentine Resolution 173/15—New Breeding Techniques.[456] Brazil made a similar determination in August 2019.[450] Alternatively though, AquAdvantage salmon, also produced through AquaBounty, is a transgene organism. This salmon

(*Salmo salar*) contains a rDNA construct made up of the growth hormone gene from Chinook salmon (*Oncorhynchus tshawytscha*) controlled by a promoter from ocean pout (*Zoarces americanus*). This genetically engineered salmon is capable of reaching market size in about half the time as conventional Atlantic salmon.[457,458] Due to the lack of prior examples, the process of regulatory approval necessary for these fishes to become marketable was very extensive. For example, it took 26 years after applying to the US Food and Drug Administration before the AquAdvantage salmon was approved.[459]

Despite headway made within regulatory acceptance of genetically modified food, government regulation and public acceptance remain one of the major barriers towards the full integration of gene editing within the aquaculture industry. Generally, society is more accepting of gene editing that results in an edit that is indistinguishable from natural mutations and is indistinguishable from an organism produced through conventional crossbreeding and backcrossing.[460] Additionally, consumers are more accepting of products where companies are transparent with the reasons why the organisms were gene edited and what methods had been used. Recently, a study was conducted to determine the acceptability of gene-edited foods among Japanese consumers.[461] The results of this study emphasise the importance of product transparency upon public acceptance of gene-edited food for consumption. Upon viewing an informative video on gene-edited food, acceptance of these foods being commercially available was found to be increased.[461] Developing a greater awareness of the science and technology used to create gene-edited foods as well as the benefits that can be derived from these products is expected to help increase overall consumer acceptance.[462]

## 8 | CONCLUSION

The aquaculture industry is expected to play an integral role in meeting future nutrition demands and addressing global food security. Even though aquaculture is one of the fastest-growing food sectors, there remains dramatic room for genetic improvement. The incorporation of genomic technologies to better assist selective breeding decisions has led to major improvements within broodstock breeding values in a much shorter period of time compared to standard selective breeding techniques; this incorporation has led to an average increase in the prediction accuracy by 22% for disease resistance traits and 24% for growth-related traits.[9] However, the further incorporation of gene editing has the potential to expedite genomic improvement through the fixation of desirable alleles at a much faster rate. Furthermore, recent advances in genomic technologies have allowed researchers to pinpoint certain genes that are responsible for the development of commercially advantageous traits; although, research has been biased towards a select number of species (Figure 4). Future work should expand on the application of these gene editing technologies in understudied species, especially in cases targeting standard physiological processes shared across species. An important, intermediate step for the use of gene editing for trait selection should be the incorporation of gene editing in piscine cell lines before application in whole organisms. Preliminary testing could assist in the selection of CRISPR construct design, may help realise putative causative gene(s), and allow for a better understanding of growth, function and overall health.[463] Compared to gene editing in whole organisms, testing cell lines is cost-efficient, offers faster results and eliminates the constraint of limited fish embryos.

Several knowledge gaps remain when it comes to targeting commercially advantageous traits through the use of gene editing technologies. Applying genomic technologies to select for traits influencing sexual development should be prioritised to reduce generation interval and improve the overall efficacy of trait selection, as well as prevent the negative connotations associated with the use of hormones in the food sector. Although much progress has been made in the identification of certain genes that influence sexual determination and differentiation (Figure 5a), results are not always consistent between studies. Moreover, inducing sterility within aquaculture species will likely become a common practice in order to address interactions between escapee fish and wild stock, as well as prepare for the application of surrogate broodstock technologies in selective breeding programs. The use of surrogate broodstock technologies can be used to shorten the interval at which highly beneficial gene edits can be disseminated throughout a breeding population, as well as allow for the production of progeny in species that are easier to rear in captivity, or reproductively mature at a younger age. Surrogate broodstock technology will likely become a common practice due to all of the benefits that may be derived. In terms of targeting traits related to growth, the majority of research has focused on the myostatin gene (Figure 5c). Next, focusing efforts to better characterise the genetic basis of traits related to metabolism and energy homeostasis should follow in order to further increase somatic growth and feed conversion efficiency. Targeting specific traits for body colouration is difficult since pathways regulating pigmentation composition can vary greatly from species to species. A greater understanding of the cellular pathways involved in the generation of pigmentation cells is necessary to make informative decisions on a larger scale. In terms of disease resistance, the incorporation of preliminary studies in cell culture, especially utilising techniques such as GeCKO, will be essential. Having a wide range of immortal piscine cell lines available for research would help streamline diagnostic capabilities. In the future, these results can be incorporated through gene editing technologies to conceivably reduce the subsequent impact of any disease outbreaks caused by the pathogen. The further development of gene editing technologies may allow for complex gene editing that is capable of targeting multiple loci/chromosomal locations to target complex traits and/or target multiple traits to help reach the multiple breeding goals genetic improvement programs have set.[112,464]

The incorporation of gene editing within aquaculture production is currently at the research stage and incorporation within aquaculture breeding programs remains within its infancy. Research still needs to focus on further developing methods to disseminate gene edits into fish production. Nonetheless, the acceptance of gene-edited fish on the market signifies a step forward in terms of both regulatory

approval and public acceptance. In the meantime, it is important to remain cognisant and updated on current policies regulating the incorporation of gene-edited organisms within the aquaculture industry. In terms of regulatory decision making, it is becoming more acceptable to consider the end product as opposed to the process used to develop a product, thereby putting a greater focus on the safety of the final product in terms of human consumption as well as the health of the organism itself.[447] Additionally, since the regulation of gene-edited organisms depends highly upon environmental protection legislation, the demonstration of effective confinement of gene-edited organisms is also highly important,[450] and incorporation of gene editing to induce sterility may also be desirable to help address this impediment. Finally, there should be an increased effort to better inform consumers on the benefits of gene-editing within the aquaculture industry to further promote the acceptance of these types of organisms on the market. Society tends to be more accepting of gene editing when the end product is indistinguishable from a product created from conventional crossbreeding and backcrossing.[460] In conclusion, the incorporation of gene editing technologies within the aquaculture industry has shown to have great potential for identifying specific genes related to commercially desirable traits as well as expediting genomic improvement through the rapid fixation of selected alleles.

## AUTHOR CONTRIBUTIONS

**Megan N. Moran:** Methodology; writing – original draft; data curation; conceptualization. **D. B. Jones:** Writing – review and editing; conceptualization; validation; project administration. **S. A. Jensen:** Writing – review and editing; conceptualization; validation; project administration. **R. Marcoli:** Writing – review and editing; conceptualization; validation; project administration. **D. R. Jerry:** Writing – review and editing; conceptualization; validation; project administration.

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## CONFLICT OF INTEREST STATEMENT

The authors do not have any conflicts of interest.

## DATA AVAILABILITY STATEMENT

Since this is a review paper, there is no data available. All information can be found in the cited references.

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## APPENDIX A: SEARCH SUMMARY

| Reproduction and Development | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Search Strings** | **Excluded in search** | **Database** | **Date** | **Period** | **Results** | **Review** | **Selected Articles** |
| "ZFN" and "aquaculture" and "reproduction" or "development" | Magazines, news articles, patents, PhD theses, Master theses, conventional breeding, health research, disease, basic research, gene expression, muscle, colour | Google Scholar | 26/04/2023 | 1995-2022 | 11626 | 2787 | 96 |
| "TALEN" and "aquaculture" and "reproduction" or "development" | | Web of Science | 26/04/2023 | All | 65 | 27 | 17 |
| "CRISPR" and "aquaculture" and "reproduction" or "development" | | | | | | | |
| **Growth** | | | | | | | |
| **Search Strings** | **Excluded in search** | **Database** | **Date** | **Period** | **Results** | **Review** | **Selected Articles** |
| "ZFN" and "aquaculture" and "growth" or "muscle" | Magazines, news articles, patents, PhD theses, Master theses, conventional breeding, health research, disease, basic research, gene expression | Google Scholar | 26/04/2023 | 1995-2022 | 9216 | 2261 | 49 |
| "TALEN" and "aquaculture" and "growth" or "muscle" | | Web of Science | 26/04/2023 | All | 44 | 16 | 7 |
| "CRISPR" and "aquaculture" and "growth" or "muscle" | | | | | | | |
| **Pigmentation** | | | | | | | |
| **Search Strings** | **Excluded in search** | **Database** | **Date** | **Period** | **Results** | **Review Articles** | **Selected Articles** |
| "ZFN" and "pigmentation" and "aquaculture" or "fish" | Magazines, news articles, patents, PhD theses, Master theses, conventional breeding, health research, disease, basic research, gene expression | Google Scholar | 26/04/2023 | 1995-2022 | 12470 | 1254 | 43 |
| "ZFN" and "aquaculture" and "pigmentation" or "skin colour" or "body colour" | | Web of Science | 26/04/2023 | All | 32 | 11 | 9 |
| "TALEN" and "pigmentation" and "aquaculture" or "fish" | | | | | | | |
| "TALEN" and "aquaculture" and "pigmentation" or "skin colour" or "body colour" | | | | | | | |
| "CRISPR" and "pigmentation" and "aquaculture" or "fish" | | | | | | | |
| "CRISPR" and "aquaculture" and "pigmentation" or "skin colour" or "body colour" | | | | | | | |
| **Disease** | | | | | | | |
| **Search Strings** | **Excluded in search** | **Database** | **Date** | **Period** | **Results** | **Review** | **Selected Articles** |
| "ZFN" and "aquaculture" and "disease resistance" or "innate immunity" | Magazines, news articles, patents, PhD theses, Master theses, conventional breeding, basic research, gene expression | Google Scholar | 26/04/2023 | 1995-2022 | 2002 | 636 | 12 |
| "TALEN" and "aquaculture" and "disease resistance" or "innate immunity" | | Web of Science | 26/04/2023 | All | 24 | 13 | 4 |
| "CRISPR" and "aquaculture" and "disease resistance" or "innate immunity" | | | | | | | |

**FIGURE A1** Search summary for the most commonly targeted traits relevant to gene editing in aquaculture.

## APPENDIX B: GENE DEFINITIONS

| | |
|---|---|
| *Acvr2* | activin A receptor, type II |
| *Alb* | albumin |
| *Amh* | anti-Müllerian hormone |
| *Amhr2* | anti-Müllerian hormone receptor type 2 |
| *Asip1* | Agouti-signalling protein 1 |
| *Bco* | Beta-Carotene Oxygenase 1 |
| *Bid* | BH3 interacting domain death agonist |
| *Csf1r* | colony stimulating factor 1 receptor |
| *Cyp19a1a* | cytochrome P450, family 19, subfamily A, polypeptide 1a |
| *Dct* | dopachrome tautomerase |
| *Dmrt1* | double sex and mab-3 related transcription factor 1 |
| *Dnd* | dead end gene |
| *Edn3* | endothelin 3 |
| *EGFP* | enhanced green fluorescent protein |
| *Esr2a* | estrogen receptor alpha |
| *Esr2b* | estrogen receptor beta |
| *Foxl2* | forkhead box transcription factor L2 |
| *Gata2* | GATA binding protein 2 |
| *Gch* | GTP cyclohydrolase I |
| *Gol* | same as *Slc24a5* |
| *Gpr54* | G-protein-coupled receptor for kisspeptin |
| *Hps4* | Hermansky-Pudlak syndrome-4 |
| *Igf3* | insulin-like growth factor 3 |
| *JAMA* | F11 receptor |
| *Kiss* | kisspeptin protein |
| *Kit* | gene that provides instructions for making a member of a protein family called receptor tyrosine kinases |
| *Kitlga* | kit ligand a |
| *Lepr* | leptin receptor gene |
| *Mavs* | mitochondrial antiviral signaling protein |
| *Mc1r* | melanocortin 1 receptor |
| *Mc4r* | melanocortin 4 receptor |
| *Mitf* | microphthalmia-associated transcription factor |
| *Mpv17* | mitochondrial inner membrane protein MPV17 |
| *Mstn* | myostatin |
| *Nanos2* | nanos C2HC-type zinc finger 2 |
| *Nanos3* | nanos C2HC-type zinc finger 3 |
| *Ntl* | no tail gene |
| *Pax7* | paired box 7 |
| *Phd3* | egl-9 family hypoxia inducible factor 3 |
| *Pmel* | premelanosome protein |
| *Pnp4a* | purine nucleoside phosphorylase 4a |
| *Pomc* | proopiomelanocortin |
| *Pts* | 6-Pyruvoyltetrahydropterin Synthase |
| *Rrm1* | ribonucleotide reductase catalytic subunit M1 |
| *Rspo1* | R-spondin-1 |
| *Scarb1* | scavenger receptor class B member 1 |
| *Sirt5* | sirtuin 5 |
| *Slc24a5* | solute carrier family 24 member 5 |
| *Slc45a2* | solute carrier family 45 member 2 |
| *Slc7a11* | solute carrier family 7 member 11 |
| *Spr* | sepiapterin reductase |
| *Stat5.1* | signal transducer and activator of transcription 5 |
| *Tyr* | tyrosine |
| *Tyrp1* | tyrosinase related protein 1 |
| *Tyrp2* | tyrosinase-related protein-2 |
| *Xdh* | xanthine dehydrogenase |

**FIGURE B1** Full names of genes referred to in review.