Trends in Genetics

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Review

Potential of Genome Editing to Improve Aquaculture Breeding and Production

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Aquaculture is the fastest growing food production sector and is rapidly becoming the primary source of seafood for human diets. Selective breeding programs are enabling genetic improvement of production traits, such as disease resistance, but progress is limited by the heritability of the trait and generation interval of the species. New breeding technologies, such as genome editing using CRISPR/Cas9 have the potential to expedite sustainable genetic improvement in aquaculture. Genome editing can rapidly introduce favorable changes to the genome, such as fixing alleles at existing trait loci, creating *de novo* alleles, or introducing alleles from other strains or species. The high fecundity and external fertilization of most aquaculture species can facilitate genome editing for research and application at a scale that is not possible in farmed terrestrial animals.

Highlights

Aquaculture is an increasingly important component of global food security, and there is major potential for genetic improvement to contribute to sustainable production.

The high fecundity and external fertilisation of most aquaculture species are amenable to the application of genetic improvement technologies, including genome editing using CRISPR/Cas9.

Disease resistance is a major target trait for improvement, and CRISPR/Cas9 offers new opportunities to fix existing alleles, to perform introgression-by-editing of alleles from wild populations or related species, and to create *de novo* alleles.

Combining *in vivo* and *in vitro* screening approaches has the potential to identify functional disease resistance alleles for downstream functional testing and application.

Using genome editing to achieve 100% sterility of production animals is a promising avenue to prevent interbreeding of escapees with wild stocks.

The Role of Aquaculture in Food Security

Food security is a major and increasing global challenge, associated with a rapidly growing demand for high-quality animal protein. Competition for land use will present a serious limitation to the scope for increases in terrestrial crop and animal production [1,2]. Therefore, it is likely that aquaculture will have a growing role in meeting this rising food and nutrition demand. Fish production via aquaculture is now approximately equal to capture fishery production for the first time in history, will be the dominant source of seafood within a few decades [3], and is the fastest growing food production sector, predicted to grow by 31% over the next 10 years [4]. Fortunately, development potential is huge, with only ~1% of suitable marine sites currently being used for aquaculture [5]. Furthermore, aquaculture production is considered efficient in terms of feed conversation and protein retention compared with most terrestrial livestock [6], and seafood is the major source of long-chain polyunsaturated fatty acids, which are considered essential for human health [7]. However, relative to many crop and livestock production systems, most aquaculture is at a formative stage and is typically a high-risk activity. Sustainability can be hindered by an initial lack of control of the reproduction cycles of species, and periodic collapses due to infectious diseases. Upscaling and improving the reliability of production will require disruptive innovation in engineering, health, nutrition, and genetic improvement technologies, the latter being the focus of this review.

Genetic Improvement for Sustainable Aquaculture Production

Domestication and genetic improvement of terrestrial livestock has occurred for several millennia, with organized breeding programs for most species in place for >50 years. The results have been striking; for example, selective breeding has led to a threefold increase in efficiency of milk production in cows, with similar gains for other target traits [8]. By contrast, relatively little aquaculture production is underpinned by modern selective breeding programs [9,10]. Most farmed aquatic species are either still sourced from the wild or in the early stages of domestication, suggesting that there is substantial standing genetic variation for traits of economic importance. The reproductive biology of aquatic species can be amenable to the application of genetics and breeding technologies, enabling high selection intensity and, therefore, genetic gain. In part, this is due

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to the near-universal high fecundity of aquatic species, and the resulting large nuclear families, which can facilitate extensive collection of phenotypic records in close relatives (including full siblings) of selection candidates in breeding programs. The reproductive output from genetically improved **broodstock** (see Glossary) together with ease of transport of eggs and juveniles, also means widespread dissemination of improved stocks can have a rapid impact on production. Furthermore, with the development of high-density **SNP arrays** and routine genotyping by sequencing [11], **genomic selection** has become the state-of-the-art in several globally important aquaculture sectors, offering higher selection accuracies than selection based on phenotypic and pedigree records alone [12,13]. However, genetic progress in selective breeding is limited by the heritability of the target traits, the generation interval of the species, and the need to target multiple traits in the breeding goal. In addition, advanced breeding programs are typically closed systems, and are limited to the standing genetic variation in the broodstock (typically sourced from a limited sample of wild populations), and new variation that arises from *de novo* mutations. Genome-editing technologies, such as **CRISPR/Cas9** (Box 1), offer new solutions and opportunities in each of these areas.

Current Status of Genome Editing in Aquaculture Species

Genome editing using CRISPR/Cas9 was recently successfully applied *in vivo* and/or in cell lines of several major aquaculture species of Salmonidae (Atlantic salmon, *Salmo salar* and rainbow trout, *Oncorhynchus mykiss*), Cyprinidae (Rohu, grass, and common carp, *Labeo rohita*, *Ctenopharyngodon idella*, and *Cyprinus carpio*, respectively), Siluridae (channel and southern catfish, *Ictalurus punctatus*), as well as Pacific oyster (*Crassostrea gigas*), Nile tilapia (*Oreochromis niloticus*), and gilthead sea bream (*Sparus aurata*) (Table 1 for details and references). One major group of aquatic species where successful CRISPR/Cas9 editing has not yet been reported is shrimp (*Penaeus* sp.), which may be partly due to practical limitations, as discussed briefly below. Most studies have a proof-of-principle focus, have typically followed

Box 1. Advances in Genome-Editing Technologies: CRISPR/Cas9 as the Game-Changer

In contrast to transgenesis, which involves the transfer of a gene from one organism to another, genome editing allows specific, targeted, and often minor changes to the genome of the species of interest. Initial progress using transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) [68,70] has been largely superseded by the advent of the repurposed CRISPR/Cas9 system [71–73]. The CRISPR/Cas9 system was discovered in bacteria, and was engineered to enable easy, cheap, and efficient targeted editing of the genome. The system creates a double-strand break (DSB) at a user-defined locus, enabling imperfect or targeted repair to create alterations to the sequence of the genomic DNA [74]. The platform functions by combining an endonuclease, the most commonly used enzyme derived from *Streptococcus pyogenes* (SpCas9), and an adapter RNA in two parts, the complementary RNA (crRNA) and the transactivating crRNA (tracrRNA). Once annealed, the crRNA recognizes the target DNA sequence, which requires the presence of a protospacer adjacent motif (PAM), and the tracrRNA binds the Cas9 protein to enable targeted endonuclease activity [71]. There are then are two primary repair mechanisms, each of which can be used to introduce different types of edit to the target genome. First, the two adjacent strands of DNA can be repaired through a nonhomologous end-joining pathway (NHEJ), which is error prone and induces insertion or deletions of a few nucleotides. Second, if a repair template is present, homology-directed repair (HDR) can be used to insert desired mutations (from a single nucleotide swap to a whole chromosomal region insertion) [72,75].

Over the past few years, technical developments have made genome editing more efficient, and raised new possibilities for biological discovery. Single guide RNA molecules (sgRNA) are routinely used instead of the crRNA and tracrRNA duplex to facilitate synthesis from a polymerase III promoter (U6) [72], which simplifies the process of CRISPR/Cas9 delivery. There have also been numerous innovations that have enabled improved precision of editing, with lower off-target rates, and broadening of the range of target sites accessible via alternative Cas9 proteins (reviewed in [76]). Novel extensions of the CRISPR/Cas9 editing system now allow researchers to achieve gene activation or inhibition, without DSBs by using a 'dead' Cas9 (dCas9) fused to activating (VP64, Rel A, and Rta proteins, known as the VPR system) or inhibiting complex (dCas9-KRAB) [77–79]. Furthermore, swapping of base pairs (base-editing) from C to T with a cytidine deaminase [76] and A to G with an adenine deaminase [80] using the same inactive Cas9 (dCas9) has the potential to target almost two-thirds of human SNPs [81].

Glossary

Broodstock: group of sexually mature fish used for selective breeding.
CRISPR/Cas9: CRISPR stands for 'clustered regularly interspaced short palindromic repeats' and Cas9 stands for 'CRISPR-associated protein 9'. CRISPR sequences together with the Cas9 enzyme can be used to make targeted changes to a genome.
Genomic selection: use of genome-wide SNPs to predict breeding values of selection candidates in a selective breeding program and to help inform which individuals to select for breeding.
Introgression: targeted transfer of a favorable allele from a donor population (e.g., a wild strain or species) to a recipient population (e.g., a breeding nucleus) using backcrossing and selection, with the target of fixing the allele in the recipient population with as little as possible of the genome of the donor included.
Introgression-by-editing: achieving the goal of introgression but using genome editing to change the target allele in the recipient population to correspond to the sequence of the allele in the donor population.
Lentivirus vector: method of delivery of CRISPR/Cas9 into cells that is particularly amenable to screening approaches using genome-wide pooled gRNAs.
Mosaicism: presence of more than one genotype in one individual. Mosaicism can arise in CRISPR/Cas9 editing of embryos if editing continues at different stages of embryonic development and each edited cell give rise to a different set of cells (organs or part of).
Off-target editing: CRISPR/Cas9 cleavage and editing at an unintended genomic location, often with sequence similarity to the site targeted by the gRNA.
Phenotyping: process for measuring the observable characteristics of individuals, in this context following the editing of a target locus.
Polygenic: a trait influenced by many QTL, each with a relatively small effect on the trait.
Quantitative trait locus (QTL): a region of the genome that contributes to genetic variation in a quantitative trait.
SNP array: SNPs are single base-pair differences in DNA sequence at a specific region of the genome. The specific variants (alleles) carried by an

CRISPR/Cas9 protocols developed in model organisms, such as zebrafish (*Danio rerio*) [35], and have often targeted genes with a clearly observable phenotype to test editing success (e.g., pigmentation). The standard methodology to induce *in vivo* mutations in aquaculture species is injection of the CRISPR/Cas9 complex into newly fertilized eggs as close as possible to the one-cell stage of development. Typically, mRNA encoding the Cas9 protein is injected together with the guide (g)RNA, leading to the high efficiency of editing demonstrated in various species to date (Table 1); using the Cas9 protein in place of mRNA is also effective [25]. While most studies have used nonhomologous end joining (NHEJ) to induce mutations, homology-directed repair (HDR) has been successfully used to insert a template DNA in Rohu carp [30]. Furthermore, successful germline transmission of edits has been reported in several of the studies to date (Table 1). **Mosaicism** is common in edited animals, implying that the Cas9-induced cutting and editing continues past the one-cell stage; this is an issue to tackle with future research (see Outstanding Questions).

Target production traits for genome-editing studies in aquaculture species to date have included sterility, growth, and disease resistance. Creating sterile animals for aquaculture is desirable to prevent **introgression** with wild stock and to avoid the negative production consequences of

individual are called a genotype. A SNP array is a microarray platform that is used to provide the genotype of an individual for many thousands of SNPs dispersed throughout the genome.

Table 1. Successful Applications of CRISPR/Cas9 Genome Editing To Date in Aquaculture Species

Species	Target gene[a]	Trait of interest	Notable features	Refs
Atlantic salmon, *Salmo salar*	*tyr/slc45a2*	Pigmentation		[14]
	dnd	Sterility		[15]
	elov-2	Omega-3 metabolism		[16]
Tilapia, *Oreochromis niloticus*	*dmrt1/nanos2-3/foxl2*	Reproduction	Germline transmission	[17]
	gsdf	Reproduction		[18]
	aldh1a2/cyp26a1	Reproduction		[19]
	sf-1	Reproduction	Germline transmission	[20]
	dmrt6	Reproduction		[21]
	amhy	Reproduction		[22]
	wt1a/wt1b	Reproduction		[23]
Sea bream, *Sparus aurata*	*mstn*	Growth		[24]
Channel catfish, *Ictalarus punctatus*	*mstn*	Growth	Germline transmission	[25]
	ticam1/rbl	Immunity		[26,27]
	LH	Sterility		
Southern catfish, *Silurus meridionalis*	*cyp26a1*	Germ cell development		[28]
Common carp, *Cyprinus carpio*	*sp7a/sp7b/mstn(ba)*	Muscle development		[29]
Rohu carp, *Labeo rohita*	*TLR22*	Immunity	Homology-directed repair	[30]
Grass carp, *Ctenopharyngodon idella*	*gcjam-a*	Disease resistance	*In vitro*	[31]
Northern Chinese lamprey, *Lethenteron morii*	*slc24a5/kctd10/wee1/soxe2/wnt7b*	Pigmentation/development		[32]
Rainbow trout, *Oncorhynchus mykiss*	*igfbp-2b1/2b2*	Growth		[33]
Pacific oyster, *Crassostrea gigas*	*mstn*	Growth		[34]

[a]Full gene names: *aldh1a2*, aldehyde dehydrogenase family 1, subfamily A2; *amhy*, anti-Mullerian hormone; *cyp26a1*, cytochrome P450, family 26, subfamily a, polypeptide 1; *dmrt1*, doublesex and mab-3 related transcription factor 1; *dmrt6*, doublesex and mab-3 related transcription factor 6; *dnd*, dead end; *elovl-2*, ELOVL fatty acid elongase 2; *foxl2*, forkhead box L2; *gcjam-a*, grass carp junctional adhesion molecule-A; *gsdf*, gonadal somatic cell derived factor; *igfbp-2b1/2b2*, IGF binding protein 2b1/2b2; *kctd10*, potassium channel tetramerisation domain containing 10; *LH*, luteinizing hormone; *mstn*, myostatin; *nanos2*, nanos C2HC-type zinc finger 2; *nanos3*, nanos C2HC-type zinc finger 3; *rbl*, rhamnose binding lectin ; *sf-1*, steroidogenic factor 1; *slc45a2*, solute carrier family 45 member 2; *soxe2* SRY-box transcription factor E2; *sp7a/sp7b*, transcription factor Sp7-like; *ticam1*, toll-like receptor adaptor molecule 1; *TLR22*, toll-like receptor 22; *Tyr*, tyrosinase; *wee1*, WEE1 G2 checkpoint kinase; *wnt7b*, wingless-type MMTV integration site family, member 7B; *wt1a/b*, Wilms tumor 1 transcription factor a/b.

Box 2. Genome Editing to Induce Sterility and Prevent Wild Introgression in Atlantic Salmon: A Case Study

Most Atlantic salmon are farmed in open sea-cages, a production method that faces sustainability challenges, such as disease transmission from wild to farmed fish and vice versa, as well as escaped farmed fish impacting wild populations [82]. A possible solution to these problems is the creation and use of sterile salmon in production.

Currently, the only method available to sterilize commercial-scale numbers of salmon is triploidization [83,84]. However, triploid (infertile) salmon are generally more sensitive to suboptimal rearing environments, which can make them prone to deformities [85,86] and less tolerant to rising seawater temperatures [87].

There are two significant additional benefits of using sterile fish. First, early maturation is prevented, which avoids the associated negative phenotypes, such as reduced growth, lower flesh quality, and higher susceptibility to disease [88]. Second, sterility in production fish may safeguard Intellectual Property for the breeding companies. The gene encoding dead end (*dnd*) has been targeted to induce sterility in salmon, preventing the formation of germ cells [15]. This was done using targeted mutagenesis against *dnd* with CRISPR/Cas9, thereby creating a gene-edited sterile fish. Germ cell-free salmon will be 100% sterile and do not enter maturity [89]. Practical application of such sterile fish in breeding programs will require developments in genome editing, including knock-in, which could lead to the production of an inducible 'on-off' system for sterility. Such mechanisms have been developed for the model fish species medaka and zebrafish [90,91]. Use of this sterility technology may foster the future development of genome editing for other traits, such as disease resistance, with negligible risk of escapees interbreeding and passing edited alleles on to wild stocks.

early maturation; in this context, CRISPR/Cas9 has been used to induce sterility in Atlantic salmon and Catfish (Table 1 and Box 2). For growth-associated traits, several groups have edited the myostatin gene (famous for its role in 'double-muscled' cattle, such as the Belgian Blue), resulting in larger fish. To date, this has been performed in channel catfish and common carp (Table 1). Immunity and disease resistance have already been investigated using genome editing in Rohu carp and Grass carp, respectively, and it is expected that this area of research will flourish as a route to improving and understanding disease resistance as a key target trait for aquaculture (see later). Genome editing can also be applied to develop models for studying fundamental immunology, such as the targeted disruption of the *TLR22* gene in carp [30]. Such models can improve our fundamental understanding of host response to infection in fish and may lead to more effective treatment protocols. Along similar lines, it is plausible to use genome-editing technology to generate improved cell lines for fish species, for example by enabling more efficient production of viruses for future vaccine development by knocking out key components of the interferon pathway [37].

Some practical reasons why genome editing has such potential for research and applications in aquaculture species are the ease of access to many thousands of externally fertilized embryos, and the large size of those embryos facilitating microinjection by hand. The ability to use large nuclear families enables a degree of control of background genetic effects, with ample sample sizes achievable for downstream comparisons of successfully edited individuals with their unedited full-sibling counterparts. The ability to perform extensive '**phenotyping**' is often also feasible, for example using well-developed disease challenge models to assess resistance to many viral and bacterial pathogens during early-life stages. Finally, should favorable alleles for a target trait (e.g., disease resistance) be created or discovered, then there is potential for widespread dissemination of the improved germplasm for rapid impact via the aforementioned selective breeding programs (Box 3). In parallel, high-quality, well-annotated reference genomes are available for most of the key species [36]. A high-quality species-specific reference genome is essential for the effective design of target gRNAs with high specificity and minimum change of **off-target editing**, in particular given the relatively recent whole-genome duplication events that are features of several finfish lineages, including salmonids [38].

Applications of Genome Editing for Aquaculture Research and Production

Infectious diseases are one of the primary threats to sustainable aquaculture, with an estimated 40% of the total potential production lost per annum [39]. Due to the formative stage of

Box 3. Integration of Genome-Editing Technologies into Aquaculture Breeding and Dissemination Programs

If the public and regulatory landscape permits, genome-editing technologies are likely to be used in commercial aquaculture breeding in the coming years. However, for widespread adoption, maximal benefit, and minimal risk, it is necessary that these technologies are seamlessly integrated with well-managed selective-breeding programs. Achieving this will help ensure careful management of genetic diversity and avoidance of potential inbreeding depression. In practice, the mass delivery of CRISPR/Cas9 to edit production or multiplier animals is unlikely to be feasible, and editing entire broodstock populations to carry the desirable alleles in the germplasm is more practical. As such, inducible editing targets may be required for impacts on traits related to sterility and maturation. In addition, technology developments are required to effectively integrate multiple edits simultaneously into broodstock animals to target multiple traits, or multiple causative alleles for the same trait [46]. Thorough testing of edited animals is required to assess and exclude possibilities of unintended and potential detrimental pleiotropic effects of edits before any application in production. However, once these issues have been addressed, widespread and rapid positive impacts could be achieved, because the high fecundity of most aquaculture species may enable dissemination to production systems without the need for pyramid breeding schemes typical of terrestrial livestock species.

domestication of many aquaculture species, new selection and disease pressures in the farm environment may increase the possibility that standing genetic variation in farmed populations includes loci of major effect, which may represent potential 'low-hanging fruit' for genome editing to increase the frequency of the favorable allele. A well-known example of a major **quantitative trait locus (QTL)** affecting disease resistance is the case of infectious pancreatic necrosis virus (IPNV) in Atlantic salmon, in which a major QTL explains the majority of the genetic variation [40–43]. Marker-assisted selection, which is based on the targeted use of molecular genetic markers linked to QTL [44], has been successfully applied to markedly reduce the impact of this disease [45]. However, despite several QTL studies in aquaculture species and ample evidence for the heritability of disease resistance traits (e.g., [45]), only a handful of large-effect QTL have been detected, and most disease resistance and other production-relevant traits are underpinned by a **polygenic** genetic architecture. As such, genetic improvement of disease resistance relies on family-based selective breeding programs, augmented by the use of genomic selection, for which disease resistance has been a major focus [12,13].

The substantial opportunity for genetic improvement of disease resistance and other performance traits in aquaculture species, combined with initial success of *in vivo* genome-editing trials, opens exciting new avenues to improve aquaculture production and sustainability. There are three main categories by which genome-editing technology could be applied to make step changes in genetic improvement, and each requires different approaches to the underpinning research leading to discovery of functional alleles: (i) detecting, promoting, removing, or fixing targeted functional alleles at single or multiple QTL(s) segregating within current broodstock populations of a selective breeding program; (ii) targeted **introgression-by-editing** of favorable variants from different populations, strains, or species to introduce or improve novel traits in a population; and (iii) creating and utilizing *de novo* favorable alleles that are not known to exist elsewhere. Here, each of these avenues are discussed in turn, and a unique opportunity to harness a combination of *in vivo* and *in vitro* approaches to understand and improve disease resistance in aquaculture species is presented.

Fixing Alleles at Existing QTL

Detecting and utilizing causative variants for QTL affecting production traits is a fundamental goal of most animal breeding and genetics research, albeit with few success stories to date [46]. Simulations have demonstrated that harnessing genome editing for favorable causative alleles at multiple QTLs as part of a breeding program, has the potential to expedite genetic gain compared with pedigree or genomic selection alone (e.g., [47]). However, a major challenge for the effective application of this approach is the successful identification of causative variation underpinning

Box 4. A Pipeline for the Discovery of Causative Variants for Complex Traits of Relevance to Aquaculture Production

Shortlisting putative functional variants underlying QTL as potential targets for *in vivo* CRISPR/Cas9 will require considerable research effort, and may include: (i) gene expression comparison of animals with disparate phenotypes for the traits of interest; (ii) functional annotation of polymorphisms within genomic regions of interest; (iii) comparative genomics, including phylogenetic conservation of the region; and (iv) detection of variants impacting gene expression of candidate loci within these regions (e.g., expression QTL or allele-specific expression). The impact of targeted edits in candidate genes can be tested *in vivo* and *in vitro* using a combination of gene knockout and ultimately 'allele-swap' experiments using HDR as a template to change the unfavorable version of an allele at a QTL to a favorable version before assessing the impact on the target phenotype. This pipeline of technologies can shortlist the many thousands of candidate variants within QTL regions down to the likely causative variant. Rapid developments in genomics technologies in fish will greatly assist this process. For example, reference genome assemblies are now available for most aquaculture species, and the focus now is on improving these assemblies and in particular on functional annotation to discover functionally relevant regions (e.g., open chromatin, promoters, enhancers, etc.) [38].

QTLs, particularly those of small effect. To achieve this, a suite of genetic and genomic technologies can be applied to shortlist of candidate variants identified from large-scale genome-wide association studies (GWAS; Box 4 and Figure 1, Key Figure). The same approach could be used to remove deleterious variants that are inevitable in populations, both large-effect variants (e.g., recessive lethal mutations), and more polygenic deleterious loads [48]. However, a challenge for polygenic traits is the need to edit multiple alleles simultaneously in the same broodstock animal(s) to achieve notable impact using this approach and, therefore, development and improvement of multiplex genome-editing approaches is required.

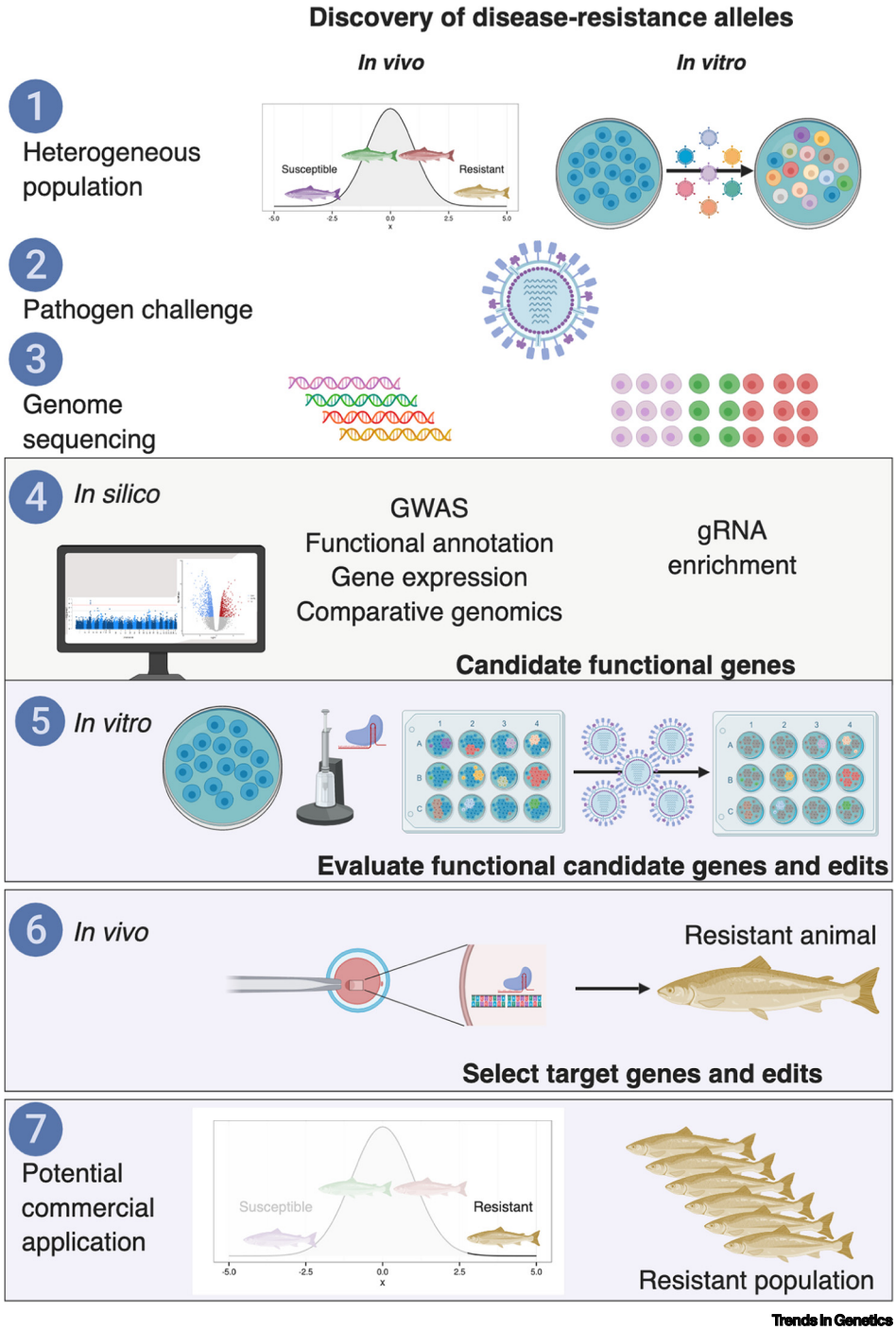
Introgression-by-Editing: Accessing Alleles from Different Strains or Species

One of the exciting possibilities of genome editing is to access genetic variation outside closed breeding populations, without the need for costly and time-consuming introgression programs, or in cases where introgression is impossible. It is common that a particular farm animal strain, or a closely related species, has a desirable characteristic. If the alleles responsible for that intra- or interspecific variation in phenotype can be identified, then CRISPR technology potentially allows editing of the unfavorable allele in the target strain and/or species to correspond to the sequence of the favorable allele found in the related strain or species (i.e., introgression-by-editing). In other words, it offers new opportunities to bypass traditional introgression, thereby avoiding the downsides associated with linkage drag (e.g., negative effects on growth rate associated with introgressing alleles from wild strains), and it allows access to genetic variation in other strains and species that would not be possible using conventional selective-breeding methods.

From a pragmatic standpoint, the early applications of such introgression-by-editing approaches will need to hold promise for transformative impacts on production to justify the extensive research and development effort required. In Atlantic salmon, parasitic copepod sea lice (*Lepeophtheirus salmonis* in the Northern hemisphere and *Caligus rogercresseyi* in the Southern hemisphere) have a crippling impact on sustainable aquaculture, with an economic impact of > GBP £700 million (USD$ 880 million) per annum globally. A unique aspect of aquaculture is the proximity of farmed species to extant wild species and populations that may have desirable characteristics. For example, certain Pacific salmon species, such as Coho salmon (*Oncorhynchus kisutch*) and pink salmon (*Oncorhynchus gorbuscha*), are largely resistant to sea lice [49,50] and are able to mount a successful immune response against the parasite. This raises the enticing possibility of transferring resistance mechanisms to Atlantic salmon, and substantial research efforts have been made to identify the factors underlying the relative differences in host resistance mechanisms, including differences in gene expression response in the iron regulation pathway, proinflammatory response related to interleukin 1 beta, or upregulation of immune receptors (e.g., C-type lectins) [51–55]. It is plausible that there are key regulatory

Key Figure

Combining *In Vivo* and *In Vitro* Screening Approaches to Identify, Test, and Apply Disease Resistance Alleles in Aquaculture Species



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genes in the pathways underpinning differential resistance between the species that could be modified in Atlantic salmon to mimic the response to sea lice exhibited by the Coho salmon. This may be targeted editing of the coding sequence and/or modulation of the regulatory sequence to enhance or suppress the expression of these key host response genes. However, temporal and/or spatial differences in gene expression often have a significant impact on a given trait, and overall modification of expression may not achieve the desired effect.

Creating *de novo* Variants Based on Knowledge of the Trait

While genome editing based on existing genetic variation (either within the farmed strain, or via introgression-by-editing) gives rise to possibilities for major benefits in animal production, creating *de novo* favorable alleles (i.e., those that are distinct from any naturally occurring alleles, to the best of our knowledge) is another exciting avenue and has already resulted in potential solutions to animal production and welfare problems. In this approach, novel alleles can be created using CRISPR/Cas9 based on *a priori* knowledge of the biology of the trait of interest, or from genome-wide genetic perturbation approaches to identify candidate genes influencing the trait. An example of the former is the development of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) resistance in pigs, where genome editing was used to knockout the *CD163* gene, resulting in a viable animal missing the entire receptor [56], or created a modified receptor by removing a specific exon and its associated protein domain [57]. Similar approaches have been used in aquaculture, including modification of the *dnd* allele to induce sterility in Atlantic salmon and targeting the *mstn1* gene in several fish species to increase growth (Table 1). Alternatively, reverse genetic screens can facilitate the discovery of *de novo* alleles impacting traits of interest. Such genome-wide CRISPR/Cas9 screens can be performed in cell lines, and may provide *de novo* targets for downstream testing and potential editing *in vivo*, in particular for disease resistance traits (see below).

Pooled CRISPR Screens for Disease Resistance

One of the most powerful techniques that emerged from the advent of CRISPR/Cas9 editing is the genome-wide CRISPR knockout (GeCKO) approach [58,59]. Briefly, this comprises creating a library of tens of thousands of gRNA to target every gene in the target organism, followed by synthesis, packaging into a **lentivirus vector**, and transduction of the cell line expressing Cas9 with a low dose aiming at approximately one gRNA integration per cell. The cell line is then screened (e.g., using a pathogen challenge) and the selected cells (surviving, fluorescently labelled, or another marker of selection) sequenced. Following selection, the enrichment or depletion of gRNAs informs the role of the target genes in the phenotype under investigation. An example of the power of this approach is the identification of the Norovirus receptor [60] using a genome-wide knockout screen in a murine cell line. This approach has been extended via the use of catalytically inactive Cas9 to enable transcriptional activation or suppression, allowing for potential gain-of-function screens in suitable cell culture systems [61].

Figure 1. *In vivo* screens: (1) start with a heterogeneous population of fish; (2) challenge with a pathogen of interest; (3) sequencing and/or genotyping of resistant and susceptible animals; (4) large-scale genome-wide association study (GWAS) approaches combined with functional and comparative genomics to detect naturally occurring disease resistance alleles in commercial aquaculture populations. *In vitro* screens: (1) pooled CRISPR knockout (GeCKO) approaches resulting in a heterogeneous population of cells; (2) followed by positive or negative selection after a pathogen challenge and; (3) sequencing to; (4) screen for enriched guide (g)RNAs can discover novel disease resistance alleles. The candidate functional genes identified by either or both approaches are then taken forward for *in vitro* (5) and *in vivo* (6) testing and characterization, then potentially commercial application, resulting in a population of animals with markedly increased disease resistance (7).

A major bottleneck for aquaculture research is the lack of suitable, well-tested, and characterized cell lines for many species of interest. Indeed, for many crustacean and molluscan species, there are no well-established immortalized cell lines. Developing such platforms will make genome-wide screening approaches a more realistic possibility in major aquaculture species. At present, CRISPR/Cas9 in cell culture is at a formative stage in fish species, albeit with promising early results. Zebrafish and medaka immortalized cell lines are readily transducible with constructs [62], suggesting that the lentivirus-mediated GeCKO approach is feasible. Recently, medaka [63], carp [31], and chinook salmon [37,64] cell lines were edited using CRISPR/Cas9. The embryonic chinook salmon CHSE-EC cell line [65] is the only fish cell line with stable integration of Cas9, another important component of pooled CRISPR screens. Various aspects of *in vitro* genome editing need to be optimized in aquaculture species, including methods for the genomic integration of large inserts and optimization of which promoter to use to drive the expression of the gRNA in the different species and systems. Viral infections (and resistance to those infections) are high-priority target traits for *in vitro* studies using CRISPR/Cas9, because the innate mechanisms of host response are usually cell-intrinsic and, therefore, amenable to interrogation in existing immortalized cell lines. Developing this technology would help facilitate an integration of large-scale genetic screens to inform the biology underpinning disease resistance and provide a pipeline of candidate alleles for application to commercial aquaculture breeding (Figure 1). The generation of Cas9 stable target animals may facilitate primary cell lines that are amenable to editing, and future development of immortalized cell lines from target tissues and/or cell types that broaden the applicability of GeCKO approaches to a wider array of aquaculture species.

Technical Challenges to Overcome

There are several important technical hurdles to be addressed to maximize the possibilities to apply genome-editing approaches in aquaculture species. First, in species where CRISPR/Cas9 has already been applied, optimization of methods is required to maximize editing efficiency, minimize off-target effects, and reduce the problem of mosaicism in the F0 generation. Off-target editing, which can result in nonspecific and unintended modification to the genome, may also lead to unintended impacts on the organism. Improved knowledge of the genome sequences of aquaculture species will assist with the design of gRNAs specific to a single targeted region, and the relatively modest cost of whole-genome resequencing can facilitate routine screening for off-target editing events. To tackle mosaicism, short half-life Cas9 proteins have been engineered to induce double-strand breaks (DSBs) only at the one-cell stage of development of the fertilized embryo [65]. Also, in F1 crosses between mosaic F0 mutants, it is possible to screen for the most commonly formed mutations to identify animals homozygous for a single mutated allele (rather than mosaic for more than one edited allele). Precise editing in the F0 generation, such as SNP exchange to change the amino acid or form a premature stop codon, can be enabled by testing of base editing approaches and HDR using templates with precise targeted changes or a short insert [66]. To rapidly select optimal gRNAs, constructs can be tested in cell culture before *in vivo* editing. Particularly for species where access to newly fertilized embryos is difficult, such as certain shrimp species, alternative methods of delivery of CRISPR/Cas9 could be tested, including sperm-mediated transfer, microinjection of unfertilized ova, and editing primordial germ cells. Another approach to enrich for edited alleles of interest is germ cell transplantation from animals with a desired edit, into multiple sterile surrogates [67]. Analogous approaches are being successfully used for editing in chickens [68], and transfer of skills and technologies from terrestrial livestock and model organisms to aquaculture species will be key to the success of this approach.

Factors Affecting Public and Regulatory Acceptance

Innovation in technology is essential to advance food production to address the increasing global demand. CRISPR/Cas9 technology has the exciting potential to contribute to the improved quantity, quality, and sustainability of seafood production globally. However, public and regulatory acceptance are key to its potential being realized. There is considerable debate about the definition of genetic modification (GM) and whether genome-editing approaches should be considered separately. If genome editing is considered separately, the different applications discussed earlier may be subject to different regulations. For example, genome editing animals could be created with just single base changes in their genome that correspond to existing polymorphisms within farmed and/or wild stocks. Alternatively, *de novo* alleles can be created that are absent in nature to the best of our knowledge (e.g., PRRSV resistance in pigs). The former may be more acceptable to the public and could feasibly be subjected to less stringent regulatory procedures. However, the ruling by the European Court of Justice that genome edited crops should be considered GM organisms is likely to hinder the commercial-scale application of genome editing in EU-farmed species [69]. However, it is noteworthy that a GM salmon (the AquaBounty salmon with a transgenic growth hormone gene) has been approved for human consumption by the FDA and the Canadian Food Inspection Agency. Furthermore, a line of tilapia derived from genome editing by the same company has been exempted from GM regulation in Argentina. It is clear there will be longstanding uncertainty about the regulation of edited animals, and the process will vary considerably in different countries. Therefore, extensive engagement with the public and other stakeholders to facilitate knowledge-driven decisions about benefits and risks of the technologies is key. From a public acceptance standpoint, it is important to consider the nature of the target traits, and whether the potential benefits stretch beyond sustainable production and profit. For example, traits such as sterility also have downstream benefits for the environment and wild stocks, and traits such as disease resistance have substantial concurrent benefits for animal welfare.

Concluding Remarks

Aquaculture is the fastest growing food production sector, is rapidly assuming greater importance than capture fisheries, and is seen as an essential component of food and nutrition security, particularly in the developing world. The use of genetics and breeding technologies in aquaculture is rapidly increasing, with the development of high-tech breeding programs for many of the world's most important aquaculture species. Most farmed aquatic species are close to wild ancestors, and this offers a major untapped resource to enhance sustainable seafood production from aquaculture. Furthermore, the external fertilisation and high fecundity of aquaculture species offer exciting opportunities for high-resolution genetics studies to understand and improve complex traits. Genome-editing technologies, such as CRISPR/Cas9, have significant potential to expedite genetic gain for production traits. Infectious disease is one of the primary constraints to aquaculture production and, therefore, a major target for selective breeding and genome-editing approaches. Host resistance to certain pathogens is a suitable trait for the use of genome-editing technologies due to the difficulty in nondestructive measurement of the trait in breeding candidates, the plausibility of utilizing cell culture genome-wide pooled CRISPR screens, and the frequent availability of early life *in vivo*-established challenge models. Different categories of genome-editing applications include: (i) discovery of causative variants underlying single or multiple QTLs affecting traits of interest, and subsequent fixation of the favorable alleles using editing; (ii) introgression-by-editing of favorable alleles into closed breeding systems from other populations, strains, or species; and (iii) creation and use of *de novo* alleles with positive effects on the trait of interest. Genome editing together with established genetic and genomic approaches enables detection and shortlisting of candidate functional variants for downstream *in vivo* validation, and potential commercial application. While several outstanding research priorities require major

Outstanding Questions

How can we optimize the efficiency of genome-editing methods in diverse aquaculture species?

Optimization of tailored microinjection protocols and reagents is required, together with investigation of alternative delivery methods. Methods to time-limit the activity of Cas9 to reduce unwanted editing throughout embryo development should be studied.

Is it possible to identify specific causative variants as targets for genome editing?

Shortlisting putative functional variants underlying QTLs as targets for editing is a major challenge, and will require considerable research effort as described in Box 3.

What is the potential for pooled CRISPR/Cas9 screening approaches in aquaculture?

Pooled CRISPR screens for genome-scale editing typically require a suitable cell culture model. In highly fecund species (e.g., marine fish or bivalve shellfish), it may be possible to consider an '*in vivo* GeCKO' approach based on similar principles to the cell culture model but delivering the gRNA library to embryos (or gametes) to enable an early-life screening experiment.

How can genome editing be applied in selective breeding programs?

Seamless integration of genome-editing technologies into breeding programs is required to ensure continuous genetic improvement and careful management of genetic diversity. Improvements in editing technologies is required to enable multiple edits in single broodstock animals, and careful assessment of any potential unintended pleiotropic effects is necessary before commercial application.

effort (see Outstanding Questions), the high reproductive output of most aquaculture species would enable potentially favorable alleles introduced into the germplasm of a well-managed breeding program (Box 3) to be disseminated at a scale and pace not feasible in terrestrial farmed animal production. Thus, subject to favorable regulatory and public perceptions, genome-editing technology has the potential to significantly transform the sustainable production of seafood through aquaculture.

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