

# Potential of genomic technologies to improve disease resistance in molluscan aquaculture

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## Abstract

Molluscan aquaculture is a major contributor to global seafood production, but is hampered by infectious disease outbreaks which can cause serious economic losses. Selective breeding has been widely used to improve disease resistance in major agricultural and aquaculture species, and has clear potential in molluscs, albeit its commercial application remains at a formative stage. Advances in genomic technologies, especially development of cost-efficient genomic selection, have potential to accelerate genetic improvement. However, tailored approaches are required due to the distinctive reproductive and lifecycle characteristics of molluscan species. Transgenesis and genome editing, in particular CRISPR/Cas systems, have been successfully trialled in molluscs, and may further understanding and improvement of genetic resistance to disease through targeted changes to the host genome. Whole organism genome editing is achievable on a much greater scale compared to other farmed species, making genome-wide CRISPR screening approaches plausible. This review discusses the current state and future potential of selective breeding, genomic tools, and genome editing approaches to understand and improve host resistance to infectious disease in molluscs.

**Key words:** Mollusc, selective breeding, gene editing, disease resistance, transgenesis, CRISPR/Cas9

## Introduction

Aquaculture plays a major role in global food security, producing 80.1 million tonnes of food in 2016. Growth in the aquaculture sector is continuing and has become an increasingly important source of seafood as capture fisheries have remained relatively stable since 1990 [1, 2]. Molluscs, including bivalves, gastropods and cephalopods represent 21.4 % of all aquaculture globally, but avenues for further development remain due to their fundamentally different biological features compared to finfish aquaculture species [1]. Their extremely high fecundity, tolerance to diverse environments, and minimal input requirements means molluscan aquaculture has major potential for efficient animal protein production [2, 3]. Bivalves such as oysters, clams, mussels and scallops provide the bulk of molluscan aquaculture, with gastropods such as abalone and freshwater snails also making a significant contribution. Cephalopod aquaculture currently has very little commercial significance, possibly due to lack of culture expertise [4] unknown optimal nutritional requirements, and difficulties associated with animal reproduction in captivity [5]. The distribution of molluscan aquaculture is mostly focused in Asia, especially China where mollusc production accounts for more than 66 % of total aquaculture output by live weight (Figure 1) [6, 7].

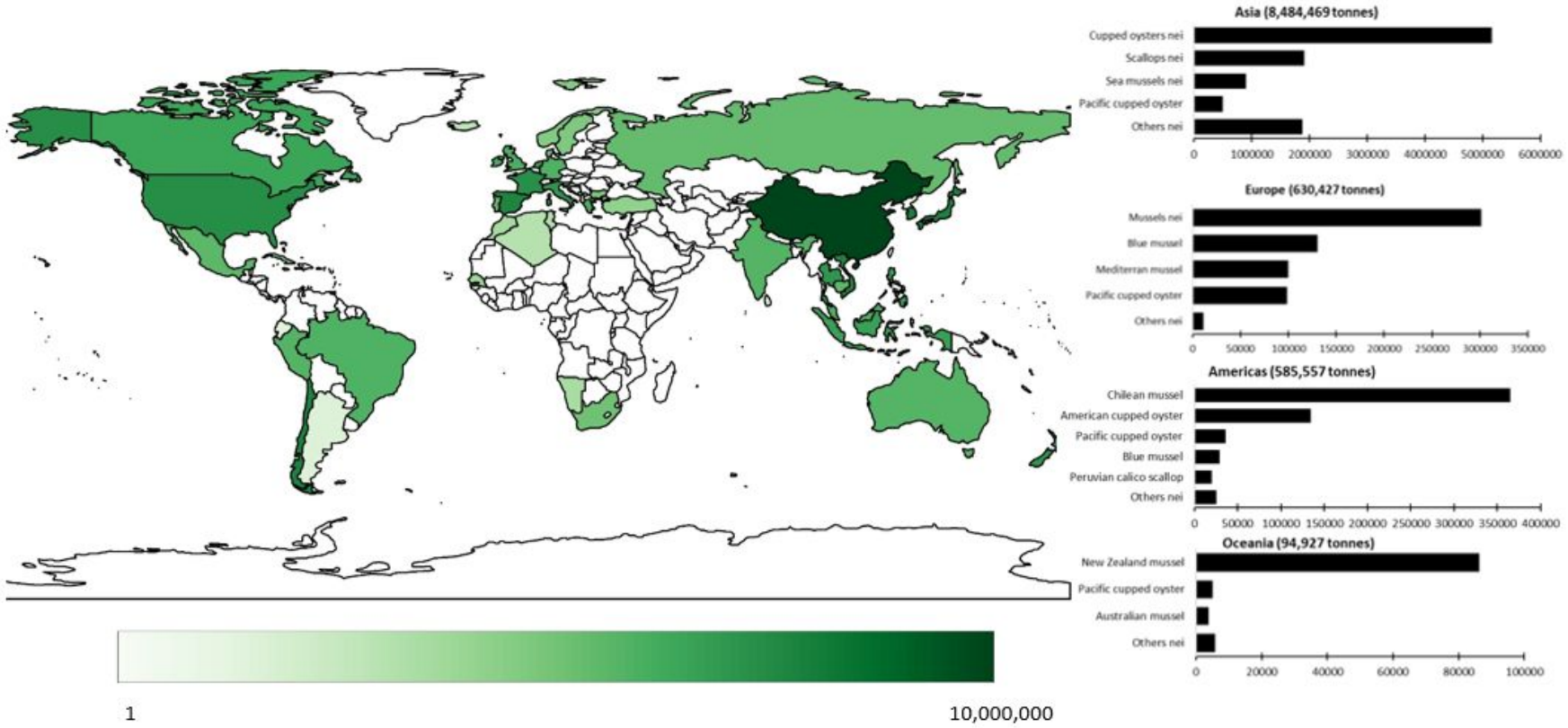


Figure 1) Global mollusc production in 2018 using FAO data (live weight tonnes $\log_{10}$). Bar charts show major production focal species across Americas, Europe, Asia and Oceania according to FAO. nei = not elsewhere included

The grow-out phase for the majority of molluscan aquaculture occurs in the open ocean environment, where animals are exposed to a wide range of viruses, bacteria, fungi, protozoans and metazoans that can cause infectious disease outbreaks [8-10]. Such disease issues can seriously constrain the sustainability and expansion of molluscan aquaculture. While assessing the impact of specific pathogens on molluscan aquaculture is challenging, one pertinent example is the devastating impact of the ostreid herpesvirus (OsHV) which is wide spread and impacts most oyster producing countries, particularly the microvariant OsHV-1 µvar [11]. Multi-faceted approaches are required to prevent and mitigate the impact of such outbreaks, especially considering potential increases in disease risk due to climate change [12]. Due to its economic and animal welfare importance, disease resistance is arguably the most important target trait in the breeding goals of advanced aquaculture breeding programmes [13-16]. Furthermore, in many cases alternative means of disease prevention are lacking, and traditional vaccination approaches are impossible in molluscan aquaculture due to open culture systems and the lack of an adaptive immune system [17].

Genetic improvement of disease resistance via selective breeding without negative consequences of reduced genetic diversity requires a well-organised breeding programme, including routine trait recording and tracking of pedigree [18]. There has been significant research effort and a number of such breeding programmes for molluscs, but the majority of molluscan aquaculture is still based on wild-sourced seed [18, 19]. Therefore, as with finfish species, there is significant untapped potential to improve production via well-managed selective breeding for genetic improvement of target traits [1, 20], which has been successfully demonstrated in molluscs [21-25]. An increasing number of genomic tools have been developed, including high quality reference genomes and single nucleotide polymorphism (SNP) arrays. These have led to new approaches to map loci affecting disease resistance, and

studies demonstrate the potential of marker assisted selection and particularly genomic selection to expedite genetic gain for target traits [18]. Genetic engineering and genome editing approaches (such as CRISPR/Cas9) have further potential to improve disease resistance, but have not yet been widely tested or applied in molluscs. Certain aspects of molluscan biology and life history traits present unique challenges and potential for genetic improvement with exciting opportunities to understand and improve disease resistance. This review will focus on the developments and applications of selective breeding and genome editing technologies to date, and discuss future innovations that are likely to have significant impacts on prevention of disease and improvement of molluscan aquaculture production in the future.

**Disease resistance traits in molluscs**

To improve disease resistance via genetic and genomic approaches, it is important to define and understand the definition of the target traits. Disease resistance is an encompassing term that from a biological perspective can describe a spectrum ranging from increased tolerance to complete protection. Both of these outcomes are beneficial to production and can be targets for improvement using selective breeding [26]. For molluscs, disease resistance in the broad sense is typically measured as the survival of animals in response to a challenge, either an experimental challenge via immersion or injection of a pathogen, or a 'field' challenge where survivors of a particular outbreak are sampled and considered as resistant. In some cases, including where the pathogen or parasite does not kill the host, counts of the pathogen per animal provide an appropriate trait to measure, with low pathogen burden considered as more resistant [27, 28].

While these measures of disease resistance are likely to be practically effective, as has been shown for finfish aquaculture species [29], understanding the molecular mechanisms underlying the resistance traits is more challenging. This is particularly the case for molluscan

species, as the mechanisms of invertebrate immunity are not as well understood as for vertebrates [30], especially given the range of host species and pathogens that impact aquaculture production. Molluscs lack adaptive immunity and instead primarily utilise non-specific responses to pathogens [17, 30]. Autophagy, phagocytosis, and other generalised defence mechanisms play a key role in the response to infection in several mollusc species [31]. Furthermore, anti-viral priming can provide an innate immunity upon reinfection, which has been demonstrated in oysters [32, 33]. However, while the cellular mechanisms of immune response and disease resistance require further research, the rapidly expanding genomic toolbox will assist in meeting this challenge (see 'Towards functional mechanisms underpinning genetic resistance in molluscs' below).

**Selective breeding for disease resistance**

Disease resistance traits have been demonstrated in several studies to be heritable, and selective breeding via family selection and mass selection has been successful in improving resistance in several oyster species (Table 1) [23]. The traits have been improved via a mix of experimental challenges and survival data collected from field outbreaks, with reasonably high genetic correlations observed between the traits measured in the two disparate environments [34], which bodes well for the use of experimental challenge models to reflect disease resistance in commercial settings. The focus of the diseases and pathogens for such studies have largely been those that cause high levels of mortalities in commercial farming, such as OsHV-1, *Bonamia spp.*(Bonamiosis), *Haplosporidium nelsoni* (MSX), and *Perkinsus marinus* (Dermo) [35-41], which can have additional negative impacts via restrictions on movement of stocks.

**Table 1)** Heritability estimates for disease resistance in molluscs since 2015 (see Degremont et al., 2015 [23])

| Host species | Pathogen | Type | Organisation / Location | Method | Heritability estimate (range) | References |
|---|---|---|---|---|---|---|
| *Crassostrea gigas* / Pacific oyster | OsHV | Industrial | MPI and Cawthron - New Zealand | Field survival and laboratory virus challenge survival of hatchery broodstock | 0.33 - 0.56 | [34] |
| *Crassostrea gigas* / Pacific oyster | OsHV | Experimental | Oregon State University - Tomales bay, USA | Field survival and laboratory virus challenge survival of hatchery broodstock imported from France | 0.22 - 0.81 | [42] |
| *Crassostrea gigas* / Pacific oyster | OsHV | Experimental | Cefas and Roslin Institute - UK | Laboratory virus challenge survival of hatchery broodstock | 0.08 - 0.25 | [25] |
| *Haliotis rufescens* / red abalone | Candidatus Xenohaliotis californiensis | Experimental | CEAZA and Universidad Católica del Norte - Chile | Measurement of disease intensity in hatchery reared from commercial stock | 0.21 - 0.36 | [27] |
| *Crassostrea gigas* / Pacific oyster | *Vibrio aestuarianus* | Experimental | IFREMER – France | Laboratory bacterial challenge of hatchery broodstock | 0 - 1.05 | [43] |
| *Pinctada fucata* / Pearl oyster | *Polydora ciliata* | Industrial | Chinese Academy of Fishery Sciences | Field challenge of hatchery broodstock | 0.21 - 0.47 | [44] |
| *Crassostrea gigas* / Pacific oyster | OsHV and *Vibrio aestuarianus* | Experimental | IFREMER – France | Field survival and laboratory challenge of hatchery broodstock at different life stages | 0 - 2.58 | [45] |
| *Crassostrea gigas* / Pacific oyster | OsHV | Experimental | IFREMER – France | Field survival and laboratory virus challenge survival of hatchery broodstock | 0.49 - 0.75 | [24] |

The significant heritability estimates for disease resistance traits highlights the potential to improve resistance in farmed mollusc stocks via breeding programmes. Indeed, a survey of mollusc breeding programmes by Hollenbeck and Johnston (2018) highlighted that various family and mass selection approaches are used across the globe , and strikingly high responses to selection have been observed for disease resistance in oysters (~16 % per generation) [18]. These results are comparable with or higher than the typical selection

responses observed for performance traits in finfish breeding, and substantially higher than for terrestrial livestock breeding [46]. There are two main potential reasons for this; firstly the high fecundity of mollusc species can facilitate a high selection intensity, and secondly the proximity of mollusc species to wild counterparts which implies significant standing genetic variation for traits important in the farmed environment. The success of both experimental and early-stage commercial breeding programmes for bivalves highlights the potential to make rapid and cumulative genetic gains for target traits [18, 22, 23].

**Breeding programme design**

Breeding programmes in molluscs must account for unique aspects of their biology and life history, which are strikingly different to livestock and finfish. The extreme fecundity of individual molluscs (up to millions of offspring per cross) provides single cross population sizes that are well in excess of what can be achieved in finfish or agricultural breeding programmes, providing potential for high selection intensity and genetic gain. Mass selection is therefore potentially a viable strategy for improving disease resistance, demonstrated by breeding from survivors of OsHV challenges that resulted in 62% higher survival after four generations of Pacific oysters [47]. Encouragingly, this improved survival was observed both in experimental challenges and in field environments, which highlights the relevance of the experimental challenge model [47]. However, mass selection is unlikely to be a sustainable approach for commercial mollusc breeding. Firstly, it is well established that mass selection can result in inbreeding, particularly in highly fecund species, and this has already been shown in oyster, abalone and scallop species [18]. Secondly, mass selection drastically limits the scope of a breeding programme to primarily focus on one specific trait of interest, losing potentially valuable genetic variation for other traits. Finally, for disease resistance in particular, challenging the candidate broodstock animals with pathogens may be a biosecurity risk, as

hatcheries should be disease free if possible, and survivors may carry and transmit the pathogen.

For most aquaculture species, it is generally considered that the cumulative genetic gains and control of inbreeding offered by family selection is the optimal approach to selective breeding [1]. For disease resistance traits in particular, tracking of individuals and families allows for experimental or 'field' challenges on siblings of the selection candidates, which enables the calculation of breeding values for those candidates without exposing them to the pathogen(s) [48, 49]. This approach is also known as sib-testing (short for sibling-testing) and harnesses the high fecundity of aquaculture species to perform disease challenges of full siblings of candidates. However, tracking individual molluscs can be challenging due to their small size and low value, particularly in the larval stages where survival rates also tend to be low. One approach is to keep families separate during early life stages, although this can confound early-life common environment with genetic effects. Genotyping approaches can be applied for parentage assignment in mixed family environments, albeit the cost of the assay needs to be low and the throughput high to be cost-effective.

While family-based selection offers the cumulative and sustainable genetic gain for production traits of interest, the use of cross-breeding to capitalise on heterosis may also be valuable. Heterosis, or hybrid vigour, has been demonstrated in *Crassostrea gigas* [50], and hybrids of different *Crassostrea* species have been experimentally demonstrated to have beneficial production values compared to single species [51]. One possible reason for the success of cross-breeding in bivalves is that broodstock lines can be relatively inbred, which may result in gains higher than would be otherwise expected [18]. Although the success of experimental heterosis suggests that underlying genetic variation in oysters harbours important traits for culture, the uptake in commercial settings has been limited [52].

Furthermore, there is currently limited evidence for the application of heterosis for traits related to disease resistance in molluscs, albeit higher general survival has been shown in some cases [53-55]. While substantial knowledge gaps remain, there may be potential to harness the beneficial effects of heterosis within the context of family-based breeding programmes, for example via the breeding and maintenance of specialist lines for cross-breeding, as commonly occurs in the pig breeding sector [56].

**Practical applications of breeding for disease resistance**

The majority of studies into the genetic basis of disease resistance in molluscs have been focused on the rock oysters *Crassostrea*, which is arguably the most important molluscan aquaculture genus globally (Table 1). Selective breeding for resistance to OsHV-1 in *Crassostrea gigas* conducted by IFREMER in France resulted in consistent improvement across 4 generations in multiple lines, with final survival gains ranging from 43.9 % to 80.6 % compared to control stocks [47]. Progeny derived from these selective breeding programmes have subsequently been used for commercial farming in various European countries [23]. Successes in developing resistance to OsHV are now being applied for resistance to *Vibrio* bacteria, which have been implicated in summer mortality [57]. This work has demonstrated that *Vibrio* resistance can be enhanced by genetic improvement, although the heritability was lower (0.09 – 0.33) than has been reported for OsHV resistance (Table 1). Additionally, little correlation between resistance to *Vibrio* and OsHV was detected which suggests that stocks selectively bred for OsHV resistance maintain potential for improvement to bacterial resistance [45]. Host age was a much more significant factor affecting resistance to either pathogen, which has been suggested previously [52, 58]. It may therefore be important to use animals of a specific age for disease challenges depending on the pathogen.

Breeding programmes for Pacific oyster on the west coast of the USA are targeting improvement in OsHV resistance/survival via family selection [42]. The federally funded Molluscan Broodstock program (MBP) has developed OsHV resistance in oysters collected from the wild in 1996. In the latest field challenges, survival varied from <10 % to >75 % depending on the specific MBP strain, demonstrating that significant gains have been made but there is still potential for improvement [59].

In Australia, a breeding programme instigated by government funded research and now overseen by Australian Seafood Industries (ASI) focuses on genetic improvement of Pacific oysters, and particularly resistance to Pacific oyster mortality syndrome (POMS) caused by OsHV-1. In this programme, 40 to 50 families of oysters are produced each year which are then subjected to both between and within-family selection, in order to maximise genetic gains whilst minimising the impact of inbreeding [60]. Finally, in New Zealand, the Cawthron Institute has run a selective breeding programme for Pacific oysters since 1999. Outbreaks of OsHV in 2010 lead to production of 49 full-sib families in 2013, which descended from survivors to OsHV-1 exposure in the natural environment. These families have formed the basis for the ongoing breeding programme to develop disease resistance in very young spat [22, 61]. In this case, breeding from survivors of family-tracked material has been highly effective, and will capture both within and between family genetic variation in resistance. However, this may not be possible in some breeding programmes where avoiding exposure of broodstock to pathogens is desirable for biosecurity reasons, as is common for aquaculture breeding [13, 15, 48].

While OsHV-1 has been the primary disease resistance target of practical selective breeding programmes to date, resistance to other pathogens and parasites has been targeted for other bivalve species. Dermo and MSX (caused by waterborne parasites *Perkinsus marinus* and *Haplosporidium nelsoni*, respectively) are persistent and highly damaging diseases impacting

Eastern oyster (*Crassostrea virginica)* production in the USA, where there have been long standing programmes to improve resistance [62]. Survivors of natural outbreaks were used to generate lines of locally resistant oysters, but as individuals were likely exposed to multiple pathogens, it is unclear to what degree resistance to each specific pathogen has been achieved. The resulting lines have been experimentally tested across the range of eastern oyster production and survivorship was generally superior compared to wild oysters, although oysters had greater survival in areas where they were selectively bred making comparison more difficult [63, 64]. Laboratory challenge of oysters with *Perkinsus marinus* revealed that there was a 54.1 % difference in survival between two families in 2014, but no significant family effect was observed when the challenge was repeated in 2015 [65]. This demonstrates that whilst the breeding programmes based on mass selection can achieve significant results, it is more difficult to develop understanding of disease resistance as environmental factors are highly variable.

Beyond *Crassostrea*, promising results from other molluscan aquaculture species include study of genetic variation in resistance and predicted selection response for withering syndrome disease (*Candidatus Xenohaliotis californiensis*) resistance in abalone *Haliotis rufescens* [27], *Listonella anguillarum* resistance in Zhikong scallop *Chlamys farreri [66],* resistance to multiple *Vibrio* species in clam *Meretrix meretrix* [67], and identification of resistance biomarkers for winter mortality/QX protozoan disease caused by *Martelia sydneyi* in *Saccostrea glomerata* [68, 69]. To date, disease resistance is not a major target for mussel breeding, when compared with other molluscs, with no published examples of genetic improvement programmes targeting disease resistance.

While the aforementioned examples of family based breeding programmes have been successful for improving disease resistance in marine molluscs, genomic tools offer many

opportunities for improving breeding programmes, including increased genetic gain. However, this requires a suitable suite of genomic tools to support these activities.

## Genomic tools and features

Reference genomes are the basis of modern genomics, as they reveal the structure, organization and functional features of genes, and provide a foundation for molecular and genomic studies [70]. High-quality reference genomes are a useful resource to advance genomic techniques for the genetic improvement of a species, as they facilitate design of genotyping platforms, discovery of molecular genetic variants, and understanding of the genetic architecture of production traits via genome-wide association studies (GWAS). Furthermore, accurate and complete genomes are essential for accurate gene editing techniques (e.g. CRISPR/Cas9) to precisely target genomic loci and prevent off-target editing.

Among commercial bivalve species, draft genomes are currently available for the Pacific oyster *Crassostrea gigas* [71], the Pearl oyster *Pinctada fucata* [72, 73] [74], the Yesso scallop *Patinopecten yessoensis* [75], the Philippine horse mussel *Modiolus philippinarum* [76], the Mediterranean mussel *Mytilus galloprovincialis* [77], the Eastern oyster *Crassostrea virginica* (Genbank Accession Number GCA_002022765.4), the Sydney Rock oyster *Saccostrea glomerata* [78], and the king scallop *Pecten maximius* [79].

In general, bivalve genomes are challenging to assemble because of their high levels of heterozygosity and repetitive DNA, particularly when short sequencing reads are being used. The problem of assembling highly polymorphic genomes with short read data is that standard genome assemblers often fail to recover the different haplotypes at heterozygous regions, reporting them as alternative contigs instead [80]. As a result, the assemblies of species with highly heterozygous genomes tend to be highly fragmented and have an artificially inflated size [81, 82]. Furthermore, bivalve genomic architecture has revealed a high prevalence of active transposable elements, which has made assembling accurate mollusc genomes difficult

due to high levels of repetition [71, 83]. Inbred individuals have been used for genome sequencing to try to mitigate the issues caused by high levels of heterozygosity [75]. However, this has not always resulted in the desired reduction in heterozygosity [71], and, could potentially reduce the range of genetic variation captured by the reference genome. Therefore, approaches to create phased, haplotype-aware genome assemblies are likely to be particularly useful for molluscs.

Third generation long-read sequencing technologies such as PacBio, Nanopore and Omni-C present a promising opportunity to improve bivalve genome assemblies, as they will allow resolution of complex regions [84]. Increased read length can potentially span the entirety of repetitive regions, which can prevent issues where the number of repeats cannot be resolved by short reads. Indeed the quality of mollusc genomes and gene annotation is already benefiting from this improved technology [79]. Importantly, the application of these technologies in combination with diploid-aware software can enable haplotype-phased assemblies [85, 86]. Resolving the diploid genome into haplotypes would provide a more accurate representation of the polymorphic landscape of bivalve genomes, facilitating downstream analysis. Mollusc genomes assembled with long read data currently include Snout Otter Clam *Lutraria rhynchaena* [87], black-shelled Pacific oyster *Crassostrea gigas* [88], the closely related *Crassostrea hongkongensis* [89], razor clam *Sinonovacula constricta* [90], blood clam *Scapharca broughtonii* [91], and Pacific oyster (Genbank Accession Number ASM1103280v1[92].

Other key genomic tools underpinning advances in selective breeding are related to high-throughput generation of genome-wide genetic markers, particularly single nucleotide polymorphisms (SNPs). This is typically performed using SNP arrays or genotyping by sequencing (GBS) approaches, each of which has advantages and disadvantages [93]. While

SNP arrays are more amenable to high-throughput genotyping of large numbers of individuals, they require significant up-front investment which tends to only be available for species with large production value and associated research communities. To date, in marine molluscs, SNP arrays are only available for Pacific and European flat oysters (Gutierrez et al. 2017, Qi et al. 2017). GBS approaches such as RAD-Seq or similar restriction enzyme-based approaches have been more broadly applied in molluscs to generate genome-wide SNP data [94], and this is partly due to its suitability for species for which very few genomic resources currently exist [95]. Both SNP arrays and GBS approaches are suitable for informing stock management, selection of animals to form base populations for selective breeding, and for assessment of the utility of genomic data in selective breeding programmes [20].

**Genomics-enabled selective breeding**

Genomic tools can improve genetic gain in molluscan selective breeding programmes via two main methods. The first is marker-assisted selection (MAS), whereby genetic markers linked to quantitative trait loci (QTL) affecting production traits are identified, and then subsequently genotyped to inform the choice of selection candidates. While this approach is likely to be successful for major-effect QTL, the majority of disease resistance traits tested in aquaculture species to date are underpinned by a polygenic architecture. The second approach is genomic selection, whereby the identification of specific QTL is less important, as genome-wide markers are used in the estimation of genomic breeding values (GEBVs) for selection candidates [96]. Initial studies testing genomic selection have shown promise in a multitude of aquaculture species (Zenger et al. 2018), including Pacific oysters [97, 98]. For example, genomic prediction of resistance to OsHV in Pacific oysters was substantially more accurate than using family or pedigree information alone [49]. Genomic selection can also be used in conjunction with so-called ‘sib-testing’ breeding designs, whereby full siblings of selection candidates are challenged with a pathogen, with mortalities and survivors being collected and

genotyped to inform on disease resistance. This allows accurate prediction of breeding values without collection of trait data on selection candidates themselves, and as such can help ensure biosecurity of the breeding nucleus as is commonplace in advanced finfish breeding programmes [48]. A major issue with genomic selection in marine mollusc breeding is that genotyping many individuals (selection candidates and reference populations) is expensive relative to the value of the animals. Therefore, approaches to reducing costs of genotyping are needed, such as genotype imputation whereby parents are genotyped at high density, and offspring at low density, then having genotypes imputed to high density [99].

**Towards functional mechanisms underpinning genetic resistance in molluscs**

While genomic selection does not require knowledge of the mechanisms underlying heritable variation in resistance to disease, there are several reasons why identifying the causative genes and variants is valuable in molluscs. Firstly, such genes and variants form potential targets for genome editing approaches to alter and fix disease resistance alleles in breeding populations. Secondly, they can help to inform alternative disease control strategies (e.g. immune priming, biosecurity). Thirdly, they can be used to improve genomic selection via applying differential weighting on functionally-relevant variants [100]. Application of SNP arrays and GBS for understanding the genetics of disease resistance are underway [25], and the typical first stage is identification of loci associated with the trait using genome-wide association studies (GWAS) [18, 93]. While disease resistance traits tend to be relatively polygenic, interesting QTL have been identified linked to genes in Pacific oyster that are relevant to viral adhesion and replication, release of viral particles, and the interferon gamma signalling pathway [25].

The regions identified by GWAS can provide targets for downstream studies investigating causative genes for disease resistance, but typically contain dozens of genes and thousands of variants. It is notoriously difficult to select candidate genes based on known literature, and

especially so in mollusc where knowledge of host response to infection is limited in comparison to better-studied farmed animals. Therefore, studies to shortlist potential functional genes and variants within the QTL region are key. These studies may take the form of a comparison of the host response to the pathogen in question between animals of alternate resistance genotypes, for example using RNA-Sequencing to assess gene expression, as has been performed for several diseases in oyster [101-103]. Overlap between differentially expressed genes and the QTL region may point to functional candidates, and QTL with cis-acting effects on gene expression (eQTL) can be used to further shortlist the candidate variants. Underpinning such research is the availability of high quality and well-annotated reference genomes. While the number of high quality mollusc genomes is increasingly rapidly, improvements in functional annotation of non-coding regions (e.g. promoters, enhancers) will greatly assist with identifying candidate functional variants [104, 105]. Finally, the rapid emergence of genome editing technologies is providing new opportunities to functionally validate such targets, for example via CRISPR/Cas gene knockout or modulation of gene expression.

## Genome editing and transgenesis in Molluscs

The CRISPR/Cas9 system is a bacterial immune response system that has been repurposed to achieve highly accurate targeted editing of metazoan genomes via double strand break and repair in DNA [106]. CRISPR based technologies are developing rapidly and have demonstrated applications in gene knock-out, insertion of exogenous DNA and editing of specific nucleotides [106, 107]. There are few examples of CRISPR editing in molluscs to date (Table 2), but gene knockout via non-homologous end joining (described in Cong et al., 2013) has been successfully demonstrated in the freshwater snail *Lymnaea stagmalis,* the squid *Doryteuthis pealeii* and *C. gigas* [108-110]. Furthermore, exogenous DNA insertion via the homology directed repair (HDR) pathway has been demonstrated in the marine snail *Crepidula fornicata* [111]. These four studies are the only reported use of CRISPR in molluscs which

demonstrates that the CRISPR/Cas9 system can be successfully implemented, but knowledge of the optimal approaches for successful editing is limited. Cell lines are typically used at early stages of gene editing research, but there are no suitable cell lines for marine molluscs and use of primary culture has significant limitations [112].

Transgenesis, the process of introducing and expressing exogenous DNA from another organism, has also been successfully demonstrated in other molluscs (Table 2). Different approaches have been successfully demonstrated, including CRISPR/Cas9 HDR, plasmid and transposon mediated transfection (Table 2). Most of these studies have been for proof of concept, only focussing on expression of reporter proteins such as fluorescence. An increase in growth rate of abalone following introduction of fish growth hormone has been reported [113], but the mechanism of this response is yet to be clarified.

**Table 2)** Summary of previous studies involving molluscan transgenesis or editing

| Reference | Species | Method | Insert | Reporter | Functional Promoter(s) | Efficiency of transgenesis (maximum) | Purpose |
|---|---|---|---|---|---|---|---|
| [114] | *Mulinia lateralis* | Pantropic retroviral vector/electroporation | LSRNL-(VSV-G) | β-galactosidase | RSV | 33% | Proof of concept |
| [115] | *Crassostrea gigas* | Particle bombardment | pDrluc and pCMVL | Luciferase | Hsp70, CMV | 0.1-0.5% | Proof of concept |
| [113] | *Haliotis diversicolor supertexta* | Electroporation of sperm | opAFP-GHc, opAFP-2000CAT | Genotyping by sequencing and southern blot analysis | SV40, AFP | 20% | Increase growth rate |
| [116] | *Crassostrea gigas* cell cultures (heart/embryo) | Pantropic retroviral vector | LLRNL, LLRLL, LN-hsp70-lucL | Luciferase | RSV, Hsp70 | 0.5% | Proof of concept |
| [117] | *Crassostrea virginica* | Electroporation | Plasmid pS65T-C1 | rsGFP | SV40, CMV | <1% | Proof of concept |
| [118] | *Mytilus galloprovincialis* | Electroporation/natural transfection | Plasmids pCMVlacZ and pMTbGH | Genotyping by sequencing | CMV, MT | Not reported (samples pooled for PCR) | Proof of concept |
| [119] | *Mytilus galloprovincialis, Mytilus chilensis* and *Chamelea gallina* | Natural transfection | p-GeneGrip | GFP | CMV | 58.5 to 70.01% | Proof of concept |
| [120] | *Haliotis diversicolor supertexta* | Microinjection into testis | Plasmid pOBA–YPGHc | None | medaka β-actin promoter | 20% | Increase growth rate |
| [121] | *Crassostrea gigas* | Electroporation | phsp-BiT-RFP-dsHOXCG1 | RFP | Drosophila HSP70, TRE, CMV | 61% | Repressible lethality |
| [111] | *Crepidula fornicata* | Microinjection of Cas9 RNA, gRNA and template for Homology directed repair | β-catenin/ mCherry | mCherry | CMV | 11% | Proof of concept |
| [122] | *Crassostrea gigas* | Electroporation of gametes/transposase | piggyBac Dual Promoter (with gGH) | GFP | CMV, EF1alpha | 0.4% | Proof of concept |
| [109] | *Crassostrea gigas* | Microinjection of Cas9 RNA and gRNA targeting myostatin gene | N/A | Genotyping by sequencing | N/A | 26.7% | Proof of concept |
| [106] | *Lymnaea stagnalis* | Microinjection of Cas9 RNA and gRNA targeting formin gene | N/A | Morphology and genotyping by sequencing | N/A | Up to 100% | Confirmation of formin gene function |
| [123] | *Haliotis discus hannai* | Microinjection of TALEN mRNA | N/A | Genotyping by sequencing | N/A | 50% | Proof of concept |
| [124] | *Crassostrea gigas* | Microinjection of plasmids | Multiple plasmid fragments | EGFP | Endogenous elongation factor1-α | 54% | Improve promotor |
| [125] | *Crassostrea virginica* | Chemical transfection of hemocytes | *p*CgVEGF-HA-IRES-GFP | GFP | CMV | 0.13% | Proof of concept |
| [110] | *Doryteuthis pealeii* | Microinjection of Cas9 nuclease and CRISPR gRNAs | N/A | Visual inspection of natural pigmentation | N/A | >90% | Proof of concept |

Abbreviations: CRISPR = clustered regularly interspaced short palindromic repeats, Cas = CRISPR associated protein, gRNA = guide RNA rsGFP = red shifted green fluorescent protein, GFP = green fluorescent protein, RFP = red fluorescent protein, RSV = rous sarcoma virus, Hsp70 = heat-shock protein 70, CMV = cytomegalovirus, SV40 = simian vacuolating virus 40, AFP = alpha-fetoprotein, MT = mouse metallothionein, TRE = tetracycline response element, EF1 = Human elongation factor-1, PCR = Polymerase chain reaction, TALEN = transcription activator-like effector nuclease

**Future perspectives on genome editing in marine molluscs**

The increasing demand for reliable production of marine molluscs makes mitigating impacts of disease essential. This can be achieved by maintaining and expanding breeding programmes to include the latest technologies to expedite genetic improvement. However, genome editing has potential to both improve selective breeding via the identification of functional variants impacting traits of interest, and via direct editing of mollusc broodstock for enhanced disease resistance in progeny. As described above, genome editing can be applied as an experimental tool in mollusc species to understand the functional basis of genes relating to disease resistance. This provides a means to improve understanding fundamental molluscan biology, in addition to the potential practical downstream benefits for aquaculture. For example, knock out of formin gene in freshwater snail *Lymnaea stagnalis* was used as confirmation of role in chirality of shell formation [108]. This can be applied to aquaculture relevant species focusing on genes relevant for disease resistance identified by genomic approaches [25, 102].

Genetic engineering approaches also have application beyond elucidating gene function in molluscs, which have certain amenable features of reproductive biology. Extreme fecundity can be used to overcome issues of poor transgenesis efficiency which can be a major problem for transgenesis / editing studies. Editing extremely large numbers will require mass delivery of CRISPR/Cas9 via electroporation or natural transfection, as opposed to microinjection of gametes (Table 2). Furthermore, high fecundity facilitates the use of high throughput disease challenge of edited larvae for rapid phenotyping of edits. Editing gametes may reduce the issue of mosaicism, as cells are edited prior to any nuclear division, but resulting edited embryos will be heterozygous for the target edit unless both eggs and sperm are edited. It may also be plausible to use sperm as the delivery vehicle for the CRISPR/Cas9 complex to edit the eggs after fertilisation, an approach shown to be successful in chickens [126]. The abundance of active transposable elements in molluscan genomes could be adapted to allow insertion of

exogenous DNA sequences, which has been successfully demonstrated in the pacific oyster to incorporate green fluorescing protein (GFP) [122]. Combined with the large number of gametes, low transgenesis and editing efficiency means there is a clear need for a high throughput screen to select for successfully edited / transgenic gametes / embryos. One possible option is to use a FACS (fluorescence activated cell sorting) based approach, where the cells ( either gametes or embryos) contain a fluorescent protein, such as GFP. Incorporating fluorescent proteins has been successful in molluscs previously through CRISPR/Cas9 HDR, electroporation and natural transfection (Table 2). This approach would need to be optimised to account for issues such as delayed time until expression of fluorescent protein, and/or the relatively large size of embryos [127]. Alternatively, antibiotic selection using G418 has been demonstrated to be a potential approach for enrichment of transfected embryos in *Crassostrea virginica* [117].

While early studies in aquaculture species have focussed on editing single genes [128], there is potential in highly multiplexed editing and screening experiments. Genome wide CRISPR knock-out (GeCKO) and synergistic activation mediator (SAM) are approaches to either disrupt or upregulate a single specific gene in one cell within a population of thousands of uniquely edited cells [129-131]. These screens involve the design of a library of tens of thousands of unique guide RNAs, typically aiming to target every gene in the organism. These gRNAs are packaged into a lentivirus vector, and transduced into cells (typically cell lines constitutively expressing Cas9) at a dose aiming for approximately one gRNA integration per cell. The edited cell line is then screened (e.g., using a pathogen challenge) and the selected cells (e.g. surviving cells or those expressing a fluorescent marker) are sequenced. The enrichment or depletion of gRNAs thereby informs on the putative role of the target genes in the phenotype under investigation. Such approaches could potentially be extended to in vivo studies in molluscs, with early life larval screens replacing the cell line. An effective high

throughput screening system combined with mass spawning of molluscs could enable development of a high throughput whole-organism genetic editing system. Larval disease challenges can be included as a high-throughput approach to identify disease resistance candidate genes for downstream study, or editing for DNA associated with resistance [25] for commercial production.

Finally, once putative disease resistance alleles have been detected or created, it may be plausible to use genome editing of commercial broodstock to breed disease resistant molluscs for aquaculture. This approach would need to be integrated within a well-managed breeding programme to ensure that there was appropriate maintenance of genetic diversity, and focus on multiple traits. Furthermore, substantial consideration would need to be given to both the practicalities of commercial-scale application, and the public perception and regulatory landscape surrounding genetic engineering and genome editing. An advantage of using CRISPR/Cas editing (rather than introduction of exogenous DNA) is that resulting animals are not transgenic (although they may be still be classified as genetically modified), and have no foreign DNA in their genomes. This is a key consideration for animals that are intended for possible production and consumption, as previously developed transgenic animals intended for human consumption (e.g. AquAdvantage salmon from AquaBounty™) has been subject to a lengthy and difficult regulatory progress [132]. However, the legal and regulatory landscape is rapidly evolving and varies substantially according to geographical location. It is important that engagement with regulators, public, and other stakeholders relating to the benefits and risks of such approaches goes alongside the technical developments and their application to mollusc aquaculture.

**Conclusions**

Disease resistance in marine molluscs has been demonstrated to be heritable and amenable to improvement via selective breeding approaches. As uptake of breeding technology increases across mollusc aquaculture, genetic improvement could be expedited by the use of the rapidly expanding genomic toolbox, via cost-effective applications of genomic selection. Furthermore, as genome editing and transgenesis approaches develop, they will facilitate identification of functional disease resistance alleles, which will both improve our understanding of disease biology, and provide potential novel avenues for commercial application. These approaches together hold substantial potential to produce molluscs with resistance to existing and future pathogens for aquaculture.

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