

Review

Review of the RNA Interference Pathway in Molluscs Including Some Possibilities for Use in Bivalves in Aquaculture

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Abstract: Generalised reviews of RNA interference (RNAi) in invertebrates, and for use in aquaculture, have taken for granted that RNAi pathways operate in molluscs, but inspection of such reviews show little specific evidence of such activity in molluscs. This review was to understand what specific research had been conducted on RNAi in molluscs, particularly with regard to aquaculture. There were questions of whether RNAi in molluscs functions similarly to the paradigm established for most eukaryotes or, alternatively, was it more similar to the ecdozoa and how RNAi may relate to disease control in aquaculture? RNAi in molluscs appears to have been only investigated in about 14 species, mostly as a gene silencing phenomenon. We can infer that microRNAs including *let-7* are functional in molluscs. The genes/proteins involved in the actual RNAi pathways have only been rudimentarily investigated, so how homologous the genes and proteins are to other metazoa is unknown. Furthermore, how many different genes for each activity in the RNAi pathway are also unknown? The cephalopods have been greatly overlooked with only a single RNAi gene-silencing study found. The long dsRNA-linked interferon pathways seem to be present in molluscs, unlike some other invertebrates and could be used to reduce disease states in

aquaculture. In particular, interferon regulatory factor genes have been found in molluscs of aquacultural importance such as *Crassostrea*, *Mytilus*, *Pinctada* and *Haliotis*. Two possible aquaculture scenarios are discussed, zoonotic norovirus and ostreid herpesvirus 1 to illustrate the possibilities. The entire field of RNAi in molluscs looks ripe for scientific exploitation and practical application.

Keywords: RNA interference; mollusc; interferon; aquaculture; norovirus; ostreid herpesvirus 1

1. Introduction

RNA interference (RNAi) has proved to be a very powerful tool in biomedicine for the investigation of gene function by gene silencing and for the control of virus by lowering mortality in various animals. Despite many papers and review articles [1] describing the pathway in invertebrates [2] and aquaculture [3,4], when the articles are examined in detail, the invertebrates examined include insects [5], crustaceans [4] and nematodes [6]. Conspicuous by their absence from such reviews are the Lophotrochozoa, which includes the molluscs and the annelids. RNAi appears not to have been widely used in molluscs despite their importance as seafood, in aquaculture, as plant pests and as intermediate hosts of important animal parasites. This review was to delimit exactly what information was available on RNAi in molluscs and where RNAi might be applied in novel situations like aquaculture.

The RNAi pathway was first discovered in plants as a gene silencing phenomena. It was later revealed in nematodes where the term RNA interference was coined [7]. It can be described simply as a series of proteins which cleave dsRNA in a sequence-specific manner and have the effect of down regulating or silencing genes. Interestingly, there appears to be a non-sequence specific component triggered by long dsRNA that confers a protective effect against pathogens. This component probably works through the genes homologous to the interferon pathway in vertebrates but all components may not be functional in all invertebrates (see later).

The most recent common ancestor of eukaryotes possessed an early RNAi system. It is clear that molluscs have a functioning, efficient RNAi pathway because the RNAi pathway has been used by a number of researchers to investigate gene function by down regulation of the genes of interest. Such studies have included cephalopods [8], bivalves [9–11], and both terrestrial [12,13] and marine gastropods [14]. Whilst the genes of the RNAi pathway in molluscs are likely to be homologous to other eukaryote pathways, differences do exist between and within phyla. At least two species of *Leishmania* [15], *Trypanosoma cruzi* [16] and many fungi [17] lack the RNAi pathway completely or most components of it. In addition, the ecdozoa appear to lack the interferon system that is linked into and triggered by dsRNA (see below). Some otherwise widespread microRNAs are missing from ctenophores, cnidarians, porifera, unicellular organisms and plants [18]. A few of the apparent differences may be due to using different names in different phyla for the same functional protein. This is due to the three main groups of scientists working on different animal models (mammals, rodents and humans; *Caenorhabditis elegans*, and *Drosophila*) with each group developing their own nomenclature for functionally homologous genes.

There has been no attempt to actually catalogue what components of the RNAi pathway have been discovered in molluscs and which components are yet to be investigated. This review identifies what homologous components have been identified in molluscs, where the major gaps in knowledge are and whether there enough data to understand if there are differences in the major phylogenetic lines of molluscs. Within the existing knowledge of RNAi in molluscs, is it possible to use this information for practical outcomes, particularly in bivalve or abalone aquaculture.

2. Interfering RNAs

2.1. MicroRNA Interference; In the Beginning?

MicroRNAs are approximately 22 nucleotides (nt) in length with 2 nt overhangs on the 3' ends [19]. They are non-protein coding RNAs that are encoded in the genome of most eukaryotes. They regulate almost all gene expression and have been especially linked to the development of animals. They are often linked to gene repression or down regulation but can enhance expression of genes by repressing repressors. One of the interesting characteristics of microRNA is that they can be slightly mismatched in complementarities to the sequence they are binding to and repression will occur. This allows microRNAs the ability to imperfectly target many genes for regulation at the same time. This is contrary to short interfering RNAs which tend to be exact compliments of their target sequence.

Five microRNA constructed as stem-loop sequences (miR-1a-1, -34a, -133a, -125b, -29b and -2a) conserved across invertebrate species were shown to be up-regulated in the foot of frozen intertidal gastropod, *Littorina littorea* and three (miR-1a-1, -34a, -29b) in the hepatopancreas [20]. Dicer was also up-regulated. This discovery of these microRNAs that are conserved and widespread across invertebrates including coelenterates, sponges, polychaetes, nematodes and urichordates allows us to infer they would be present in all molluscs and likely to be operational. In other animals, these miRNAs function as follows: miR-1 regulates heat shock protein 60 which affects the myocardium [21]; miR-34 inhibits SIRT1 which affects the cell cycle and apoptosis [22]; miR-133 negative regulates many signals including K⁺ channels [21]; miR-125 down regulates p53 tumour suppressor gene [23]; miR-29 targets many cancer associated genes [24]; miR-2 affects both neural development and apoptotic genes [25].

Primary RNAs that are going to be processed into mature miRNAs undergo a series of modifications after transcription to arrive at the functional state. Initially, the primary microRNA is transcribed, captured by a dsRNA-binding protein called Pasha (or DGCR8 in humans) (Figure 1). This complexes with Drosha, a RNase III which trims it to about 65–70 nt of mainly complementary nucleotides. Then the trimmed microRNA form a secondary stem-loop (hairpin) shape [19]. These stem-loop precursor microRNAs are exported out of the nucleus by the classic Ran-GTP dependent karyopherin, Exportin 5 which in many animals [26] has been characterised as critical [27].

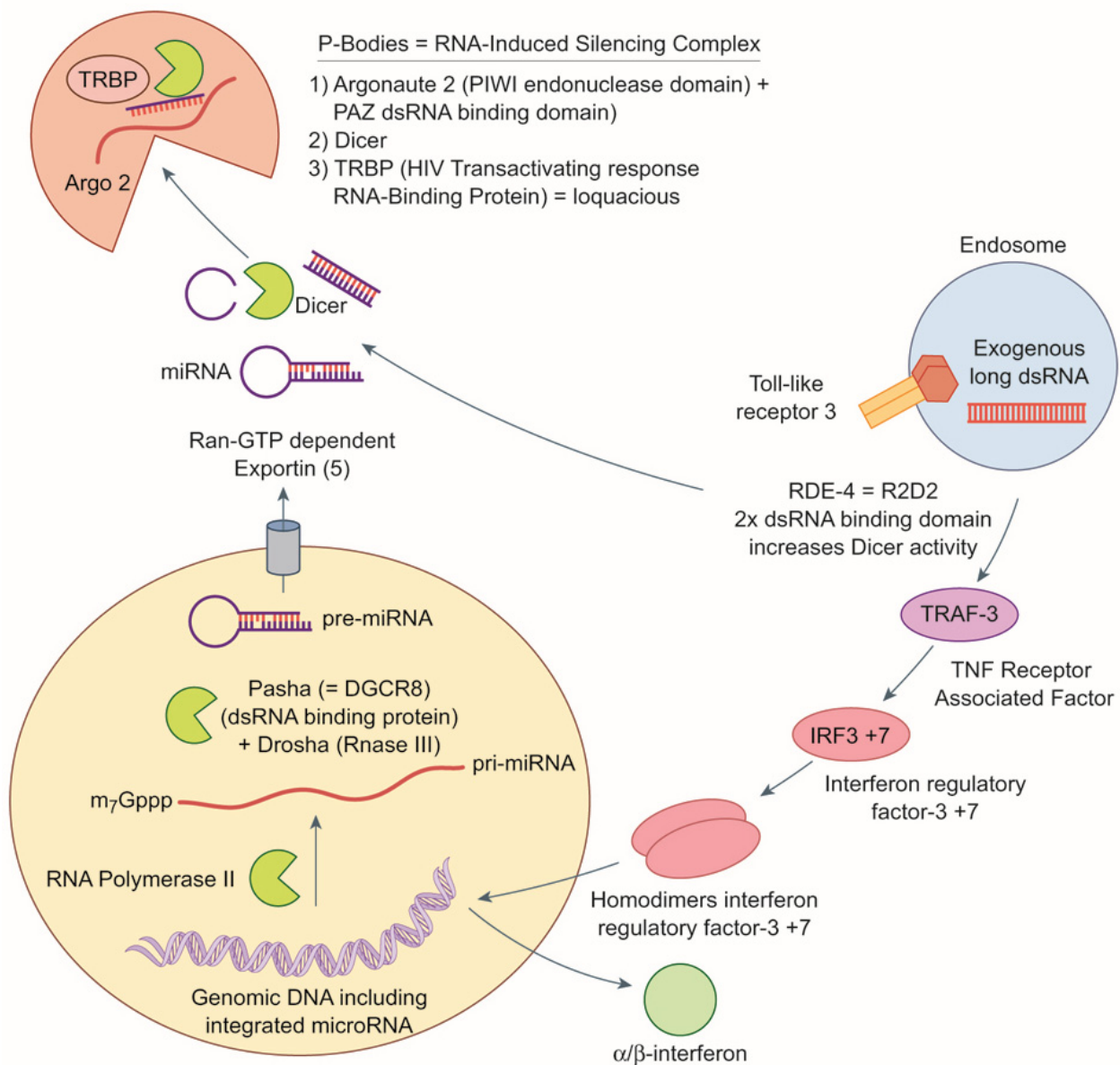


Figure 1. The components of the RNA interference pathway likely to be present in molluscs. The microRNA pathway encoded in the mollusc genome links via Dicer into the P-bodies for processing into effective RNAi. Cytoplasmic dsRNA passes from endosomes via Dicer to P-bodies stimulating the signaling α , β -interferon trans-activating γ -interferon in other cells.

Once the hairpin has exited the nucleus, it is bound by Dicer 1 ribonuclease having a dsRNA-binding site which cuts off the loop and shortens the stem to 21–25 nt with a 2 nt overhang at the 3' end of both strands. Now the RNA-induced silencing complex (RISC) or P-body forms. RISC consists of a HIV-Transactivating response RNA-Binding Protein (TRBP) (also called loquacious in insects) to capture the RNA; an endoRNase H, Argonaute 1 with a PIWI cleavage domain; and Dicer 1 containing the template RNA. One strand of the dsRNA is degraded leaving a single stranded, mature microRNA. In most animals, the complementary template mRNA binds to the RISC containing the short imperfect 21–25 nt RNA which stays bound and is called the guide strand. Argonaute 1 does not cleave the mRNA unless it is a perfect or near perfect match. Perhaps this silencing mechanism is due to the 3' microRNA

interfering with the mRNA polyA tail repressing translation of the gene's mRNA [28]. However, interference at both the mRNA 5' cap (m⁷Gppp) and the 3' ends is necessary for maximum repression [28]. In molluscs, of all the key enzymes in the RNAi system, only Dicer has been found by immunoreactivity with a rabbit anti-Dicer in the intertidal snail *Littorina littorea* [20]. However, a search of NCBI GenBank shows unpublished Dicer homologous sequences ($P < 10^{-5}$) and sequences for other key enzymes in the RNAi system in other molluscs (Table 1).

Table 1. The species of each major molluscan group that has gene sequences involved in the RNA interference pathway available on NCBI GenBank.

Enzyme in RNAi Pathway	Number in Bivalves	Number in Gastropods	Number in Cephalopods
Argonaute 2 with PIWI domain		<i>Biomphalaria glabrata</i>	
Dicer	<i>Argopecten purpuratus</i>	<i>Littorina littorea</i>	
Drosha			
HIV-TRBP = loquacious			
Interferon Regulatory Factor	<i>Crassostrea gigas</i>		
	<i>Hyriopsis cumingii</i>	<i>Haliotis discus</i>	
	<i>Ostrea edulis</i>		
	<i>Pinctada fucata</i>		
Pasha = DGCR8-like		<i>Littorina littorea</i>	
Toll-like Receptor 3	<i>Chlamys farreri</i>		
	<i>Crassostrea gigas</i>	<i>Biomphalaria glabrata</i>	
	<i>Mytilus galloprovincialis</i>		

Within the stem of one microRNA, a 21 nt sequence complimentary to 3' untranslated region called *let-7* (UGAUAUGUUGGAUGAUGGAGU) is highly conserved across most eukaryotes including molluscs but excluding ctenophores, cnidarians, porifera, unicellular organisms and plants [18]. *let-7* microRNA appears to function as the repressor of a repressor allowing the mRNA of a gene to be expressed. In the nudibranch, *Phestilla sibogae* and the polychaete, *Hydroides elegans* expression was prominent in adults but not larvae. Interestingly, a bioinformatical search in molluscs by the authors for complementary DNA transcripts of the *let-7* sequence revealed bipartite signals suggesting functionality at 10–11 nt length or splicing to produce the full 21 nt signal.

2.2. Short Interfering RNA (siRNA)

siRNA uses the same RISC/P-body components but differs from the microRNA in a number of ways. siRNA is always cytoplasmic being induced by internalisation of dsRNA most often through the plasma membrane associated with SID-1 (systemic RNAi defective) conducting channels [29]. siRNA induction is via long dsRNA that are bound by R2D2 in *Drosophila*, homologous to RDE-4 in *C. elegans* and other nematodes which have two, in tandem, RNA-binding domains and stimulate the production of Dicer or at least stabilise it [30]. Many investigations with siRNA deliver the complementary 21–25 nt template directly bypassing R2D2/RDE-4 proteins. To function, the complementary template in Argonaute 2 should be exact, rarely one or extremely rarely, two base pairs different. The mRNA of the gene is always cleaved at one specific site, opposite and after 10 nt from the 5' end of the guide strand [31].

Long dsRNA have been preferred in intervention studies over siRNA as they are believed to work better. This is perceived to be due to the ability of multiple siRNA being able to be generated by Dicer from the long dsRNA template rather than relying on just one short match. Also, long dsRNAs have been able to generate non-sequence specific protection either by enhanced Dicer induction [30] or stimulation of γ -interferon (see below). One of the questions needed to be answered is: at what length does a siRNA become a long dsRNA (see below) in terms of activity? The answer might be; there is no difference (see 30). Furthermore, [32,33] showed that siRNA of 21 nt was also able to generate non-sequence specific protection possibly by enhanced expression of Dicer which is functioning as a non-sequence specific ribonuclease.

2.3. Long dsRNA Linked to Interferon?

Long dsRNA are processed the same way as described above for siRNA. However, if the process of internalisation involves the formation of endosomes, the dsRNA is likely to trigger the Toll-like receptor 3 (TLR3). In the Zhikong scallop (*Chlamys farreri*), five functional genes of the Toll-like pathway have been found by amino acid homology and tested by ELISA and RNAi [11]. In humans, TLR3 will trigger a non-sequence dependent antiviral response through the interferon pathway, particularly γ -interferon. TLR3 is unique amongst TLRs in that it does not function through MyD88 (myeloid differentiation primary response gene 88). Instead, homodimers of interferon regulatory factor (IRF) 3 and IRF 7 promote transcription of the α/β -interferons which signal other cells in a trans-cell manner to produce the glycoprotein, γ -interferon. Interferons are non-specific inhibitors of mRNA transcription that generally slow down rapid replicators like viruses and tumour cells and turn on many other genes of the innate pathways of immunity to eliminate pathogens. Interferons are also suspected to coat receptors blocking the uptake of viruses [34].

In invertebrates, the functional presence of this interferon pathway is clouded. Using bioinformatical searches for the conserved N-terminal DNA binding domain of IRF and the C-terminal IRF association domain 1 or 2 (IAD1, IAD2), [35] did not detect IRF genes in nematodes or arthropods but they did in molluscs with 2–7 different genes in 4 functional clusters. Those molluscs deemed positive for IRF genes included the marine gastropods, *Aplysia californica*, *Lottia gigantea* and the bivalves *Crassostrea gigas*, *Mytilus edulis* and *Mytilus californicus*. Furthermore, [36] have characterised a homologous IRF 2 (within the IRF 1 family) from the pearl oyster *Pinctada fucata*. Indeed, [37] cloned a myxovirus resistance (Mx) gene from the disk abalone (*Haliotis discus*), one of the most studied interferon type 1 (α/β)-inducing proteins known. They showed that it was functional when stimulated with the artificial viral nucleic acid analogue, poly-inosinic:cytidylic acid (poly I:C). Similarly, [38] showed poly-I:C induced an immune response against ostreid herpesvirus 1 (OsHV-1) which included up regulation of toll-like receptors, MyD88, interferon regulatory factor and protein kinase R which are indicators of a type-1 interferon response.

Furthermore, also in disc abalone, [39] demonstrated the presence of another interferon-induced gene; Gamma-interferon Inducible Lysosomal Thiol reductase (GILT) which reduces the disulphide bonds associated with the major histocompatibility complex class II restricted antigens. In addition, in the Japanese pearl oyster (*Pinctada fucata*), [34] used a recombinant feline ω -interferon to block akoya virus. In the best treatment, only 18% of oysters died from the virus whilst 100% of untreated oysters

died. Survivors showed less muscle damage and extensive repair of muscle tissues that had been damaged. However when delivered at the same dose, α -interferon which is a signaling interferon rather than an effector molecule, had no effect on oyster mortalities due to the akoya virus.

In summary, genes for the interferon pathway and interferon-induced genes exist and are functional in a range of molluscs; molluscs tolerate injection of therapeutic doses of ω -interferon and these doses prevent virus-induced mortality as does the artificial virus analogue poly I:C. Despite the paucity of direct evidence in the primary literature on molluscs of an interferon-based pathway induced via dsRNA and TLR 3, there is accumulated evidence that this pathway is functioning in molluscs.

3. Possible Practical Uses of RNAi by Mollusc-Based Industries

Whilst there is a plethora of potential applications for RNAi, this review will focus on two diseases related to the aquaculture of bivalves, one of major zoonotic potential, Norovirus and one of major epizootic potential, ostreid herpesvirus 1 (OsHV-1).

3.1. Norovirus

Noroviruses are responsible for almost half the food borne illnesses and more than 90% of the non-bacterial food borne outbreaks in the USA [40]. Noroviruses are members of *family Caliciviridae* and as such consist of non-enveloped viruses containing positive sense ssRNA. They are cosmopolitan in distribution and responsible for winter gastroenteritis epidemics worldwide. There are two main genotypes in humans, GI and GII. Noroviruses bind to carbohydrate moieties particularly the fucose residue in the gut and on red blood cells that are similar to the Lewis, H and human histo-blood group antigens (HBGA). In humans, HBGA leads to a differential susceptibility to GI norovirus. HBGA-A is the most susceptible and HBGA-B is the least [41]. However, one of the latest recognised norovirus genotypes, GII.4 discovered in Sydney, Australia was not influenced by human histo-blood group antigens as it binds to a sialic acid ligand found widespread in most tissues [42].

It would be easy to deliver RNAi against norovirus to oysters in an experimental situation that could be applied at the depuration stage. For instance, a plasmid can be designed to produce mRNA complementary to the capsid gene or the viral encoded C3 protease. This would bind to the mRNA producing a dsRNA molecule that Dicer would target for destruction. As these two genes are viral encoded genes, it would be highly unlikely to have any off-target effects. This approach has already worked in insects against a penaeid virus [43] and in penaeids [33]. Oral delivery could be by live, probiotic bacteria [43], perhaps by killed bacteria or chitosan nanoparticles [44] which have been shown to be effective in other invertebrates. A protocol delivering dsRNA via the diet [33] would be even better as these dsRNAs decay to become inactive within about four weeks [44].

For RNAi intervention to decrease the titre of the norovirus in oysters, there has to be a phase of naked RNA in the cytoplasm of the cells of the oyster. Unfortunately, this information is lacking in oysters but the implication from the literature is that oysters are only an accumulative host of the virus [45]. An elegant study including quantitative PCR for norovirus in *Crassostrea virginica* and *Crassostrea ariakensis* was undertaken by [46] but unfortunately they did not report the viral titres. Instead they used a logistic regression model which produces odds ratios. Their endpoint day was day 29 for *C. ariakensis* but as the experiment only ran for that period, it is impossible to know if that was the day of extinction

for the viral titre. However, in *C. virginica* the endpoint day was 22–25, dependent on temperature, which shows slow viral clearing but not the lack of viral replication.

If indeed oysters are only an accumulative host and not a replicative host as the literature suggests, there is another RNAi approach that could be attempted. For accumulation of norovirus, a fucosyltransferase gene (*fstf* gene) like the human FUT2 must be operational to produce terminal fucose residues to efficiently bind the norovirus. Interestingly, about 20% of humans are considered “non-secretors” if this gene is mutated. Non-secretors are almost totally resistant to norovirus [41] except for GII.4. There is no known detrimental health changes in humans for mutation of this gene as other genes like FUT1 maybe be able to compensate [47]. This gives a level of safety to consumers of oysters RNAi-treated to knockdown FUT2. Furthermore, there is only a 2% homology between bivalve and human FUT2, so it should be very easy to design a RNAi against molluscan FUT2 that does not function in humans. Therefore a RNAi targeting FUT2, which has already been proven to down regulate FUT2 in cell cultures [47], could be delivered as outlined above to depurating oysters. A bioinformatical search by the authors has identified a fucose-binding lectin gene in molluscs including oysters suggesting the pathway is present. It may enhance depuration if the *fstf* gene needs to be active in the oyster for norovirus retention. This could be tested experimentally and maybe a therapy for speeding up depuration or perhaps a mutated FUT2 could be a gene marker for resistance to norovirus in selective breeding of oysters in hatcheries.

3.2. *Ostreid Herpesvirus 1 (OsHV-1)*

Ostreid herpesvirus 1 (OsHV-1) is a worldwide virus which causes epizootic mortalities up to 100% associated with rising water temperatures [48]. OSHV-1 infects many species of bivalves including the widely dispersed and cultivated *Crassostrea gigas*. It has commonly caused summer mortality and it has become more worrisome with the rising temperatures associated with global warming. Spat (newly settled bivalves) and juveniles are particularly vulnerable with mortalities [49] within 7–10 days but infection is seen as early as 3–4 days [48]. Herpesviruses have been known in oysters since the 1960's but only really have become a problem since the advent of oyster hatcheries where many of the progeny can be infected, thus producing a noticeable epizootic. Survivors of epizootics are believed to be carriers with high qPCR titres being significantly associated with mortalities whilst low titres are not [50]. Recently in 2005, a new virulent biotype, μ var OsHV-1 arose and has spread worldwide to become a major problem since 2008 [51].

RNAi could be a solution to OSHV-1 which is highly probable to be effective over time. Viral titres in invertebrates as measured by qPCR are likely to drop by 90% when appropriate RNAi is applied [4]. The capsid gene of OsHV-1 is a likely target for intervention. The question becomes one of delivery. As the spat are infected so early in life, the spawning adults have to be targeted for RNAi. Incorporation of a plasmid with complimentary DNA to the target gene into the oyster in a probiotic bacterium would be easy, but this must be done in a hatchery. As it may be possible to produce 100% sterile triploid oysters [52] although recent studies suggest that limited reversion to normal chromosome counts is possible [53], triploidy could be coupled with the introduction of the bacterium so there would be a reduced chance of spread of the plasmid to wild oysters via gametes. Since OsHV-1 is a problem exacerbated by hatcheries, then where hatcheries are being used, they can become part of the solution. Possibly dramatically

reducing one of the sources of environmental contamination will allow wild oysters time to become more resistant as resistance to OsHV-1 has been shown to be genetically highly variable [50] and therefore likely to be highly heritable.

4. Conclusions

Relative to other animal groups, the studies of the RNA interference pathways in molluscs have been comparatively sparse and all the information known is probably based on a mosaic of about fourteen species. However, there appears to be a constant theme of homologous proteins and functioning similarities in the pathways rather than differences. The area needing most attention is the cephalopods for which there is a dearth of knowledge and may be the most likely place to find novel components. There is no data on how many slightly different enzymes in the RNAi pathway there might be in each mollusc. For instance, *Penaeus vannamei* has two different Argonautes with slightly different capabilities [54] and up to four different Dicers have been found in plants [55]. Nevertheless, it seems reasonable to predict that of all the invertebrate groups so far studied, molluscs look likely to follow the paradigms for RNAi set by other studies in more recently evolved eukaryotes. This would suggest that RNAi is likely to be a major new tool for intervention against diseases in molluscs, particularly via hatcheries for aquaculture.

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Author Contributions

LO wrote most of the manuscript. SM edited and added valuable insights into the molluscan world.

Conflicts of Interest

The authors declare no conflicts of interest.

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