SCHOOL OF BIOLOGICAL SCIENCE
FACULTY OF SCIENCE & ENGINEERING

The application of RNA interference to study the biology of the *Neoparamoeba* genus

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Thoughts to live by:

"ALL I REALLY NEED TO KNOW I LEARNED IN KINDERGARTEN"

by Robert Fulghum

"Most of what I really need
To know about how to live
And what to do and how to be
I learned in kindergarten.
Wisdom was not at the top
Of the graduate school mountain,
But there in the sandpile at Sunday school.

These are the things I learned:
Share everything.
Play fair.
Don't hit people.
Put things back where you found them.
Clean up your own mess.
Don't take things that aren't yours.
Say you're sorry when you hurt somebody.
Wash your hands before you eat.
Flush.
Warm cookies and cold milk are good for you.
Live a balanced life -
Learn some and think some
And draw and paint and sing and dance
And play and work every day some.
Take a nap every afternoon.
When you go out into the world,
Watch out for traffic,
Hold hands and stick together....."

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SUMMARY:

RNA interference (RNAi) is a natural regulatory mechanism of most eukaryotic cells that uses small double-stranded RNA (dsRNA) molecules as triggers to direct homology-dependent control of gene activity. This technique has emerged as a powerful tool for rapid analysis of gene function in non-model organisms and has the potential to identify candidate targets for intervention against diseases of economic importance to aquaculture.

With regards to amoebic gill disease (AGD) of farmed Atlantic salmon, RNAi could become an invaluable research instrument to unravel the role of proteins involved in amoeba attachment and pathogenicity, as well as to validate important treatment targets by investigating the effect of specific gene knockdown on amoeba survival and physiology. Additionally, RNAi technology could greatly assist in the elucidation of possible factors associated with the loss of virulence in certain species from the *Neoparamoeba* genus.

However, before RNAi technology can be employed in *Neoparamoeba*, it is important to consider whether members of this genus possess the required set of proteins involved in the RNAi pathway. As a result, the main purpose of the present study was to use functional and comparative genomics approaches to investigate whether functional RNAi machinery has been retained or lost in species from the *Neoparamoeba* genus. As the *in vitro* culture of the causative agent of AGD (*Neoparamoeba perurans*) has been successfully achieved only recently, most of the gene regulation assays were performed using the closely-related *Neoparamoeba pemaquidensis*, which is readily amenable to culture.

Using a *N. perurans* and *N. pemaquidensis* transcriptome database we were able to identify putative proteins containing conserved domains of RNAi-related

genes, such as Dicer and Argonaute. For *N. pemaquidensis*, the candidates' involvement in the RNAi pathway was validated by assessing their levels of expression followed the administration of dsRNA and small interference RNA (siRNA), respectively. The presence of an active Dicer in both species was also corroborated by utilizing an RNAse III assay, which showed complete degradation of dsRNA following incubation in amoeba lysate. Further evidence for the presence of an active RNAi machinery was also supported by gene silencing experiments, where *N. pemaquidensis* specific genes (β-actin and EF1α) were successfully downregulated by the administration of RNAi-trigger molecules. However, knockdown efficiency was dependent on dose, administration frequency, target gene, delivery method and RNAi molecule. Additionally, trophozoites soaked with bacterially expressed dsRNA targeting β-actin unexpectedly transformed into a cyst-like stage, which has not been previously reported in this species. Unfortunately, the attempts to employ the *Entamoeba histolytica* U6 promoter to confirm the existence of a functional RNAi pathway in *N. perurans* haven't succeeded yet.

The results altogether provide strong evidence for the presence of functional RNAi machinery in *Neoparamoeba* spp. Despite being promising, these findings are still preliminary and the reality of applying RNAi technology to develop new treatment strategies against AGD still needs further effort. Therefore, more work needs to be undertaken in order to fully elucidate the RNAi mechanisms in *Neoparamoeba perurans*.

DECLARATION:

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Paula Cristina Walger de Camargo Lima

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And last, but not least, Jai Guru Jai!!!

STATEMENT OF THE CONTRIBUTIONS OF JOINTLY AUTHORED PAPERS:

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CHAPTER 2

Lima, PC; Harris, JO; Cook, M. (2013). Exploring RNAi as a therapeutic strategy for controlling disease in aquaculture. *Fish and Shellfish Immunology*, 34(3), pp.729-743.

Authors contribution: Lima, P.C. surveyed the literature and composed the manuscript; Harris, J.O. and Cook M. contributed reviewing the manuscript structure and content.

Experimental Chapters:

CHAPTER 3

Lima, PC; Botwright, NA; Harris, JO; Cook, M. Towards the application of RNAi against Atlantic salmon Amoebic gill disease: Identifying key components of the RNAi machinery in the closely related species of the genus *Neoparamoeba* (submitted to Marine Biotechnology)

CHAPTER 4

Lima, PC; Botwright, NA; Harris, JO; Cook, M. First evidence of functional RNAi mechanism in *Neoparamoeb*a genus (submitted to Parasitology)

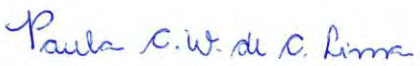
CHAPTER 5

Lima, PC; Botwright, NA; Harris, JO; Cook, M. Ingestion of bacterially expressed dsRNA induces target gene-dependent knockdown in species from the genus *Neoparamoeba* (submitted to Aquaculture)

Authors contribution: Lima, P.C. assisted with experimental design, conducted the research experiments, analysed/interpreted the data and composed the manuscripts;

Botwright, N.A. provided assistance with laboratory and analytical tasks and reviewing the manuscripts; Harris, J.O. made contribution to the drafting and revising the manuscript; Cook M. participated on assay development and results interpretation, as well as revising the manuscripts structure and content.

The following authors agree that the Statement of the contributions of jointly authored papers accurately describes their contribution to research manuscripts 1, 2, 3, and 4 and give consent to their inclusion in this thesis.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CHAPTER 1: General Introduction

1.1. Amoebic gill disease

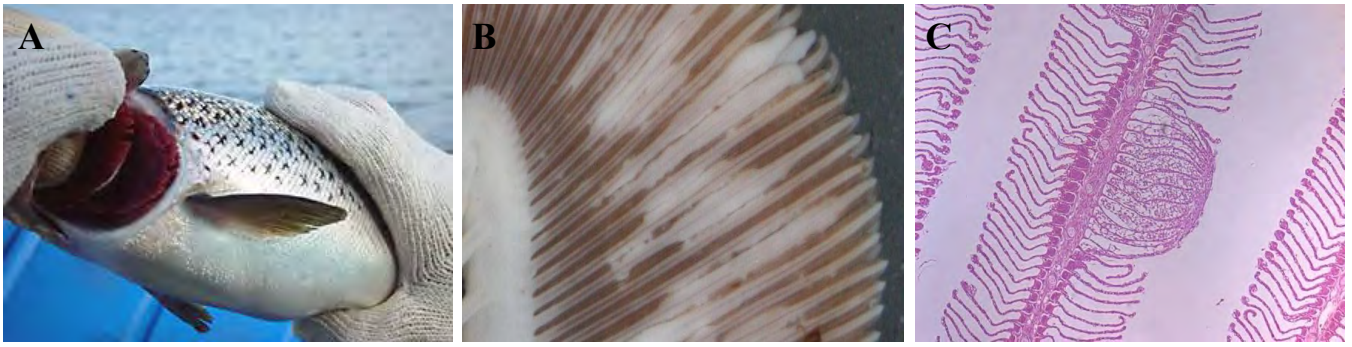
Amoebic gill disease (AGD) is a parasite-mediated proliferative gill condition capable of affecting several cultured teleost fish species [1]. Atlantic salmon appears the salmonid species most susceptible to AGD [2]. Outbreaks of the disease in this species have been reported from Australia [3], Ireland [4], France [5], Chile [6], Spain [4], United States [7], New Zealand [2], Scotland [8] and Norway [9].

Initially, *Neoparamoeba pemaquidensis* was considered the causative agent of AGD, based on morphological [10] and molecular [11] characterization of trophozoites isolated from the gills of cultured AGD-affected fish. However, another species from the same genus, *Neoparamoeba branchiphila*, was also later implicated [12], suggesting that AGD may be a disease of mixed aetiology. This hypothesis was contradicted by Young et al. [13] who demonstrated that neither *N. pemaquidensis* nor *N. branchiphila* were associated with AGD-lesions, indicating that a third species, subsequently called *Neoparamoeba perurans*, is the only confirmed aetiological agent of AGD in farmed Tasmanian Atlantic salmon.

Water salinity (35ppt) and temperature (>15°C) are the major environmental factors associated with AGD outbreaks, with amoeba starting to become apparent on the fish gill during the first summer of sea cage farming [14]. Besides the primary factors cited above, AGD has also been observed in association with a breakdown in husbandry practices, such as poor hygiene, crowding and decreased water exchange through biofouling on nets [15].

AGD is visually characterized by the presence of raised, white mucoid patches in the gill (Figure 1.1A, B). Nevertheless, full diagnosis of the disease

requires histopathology to confirm the presence of the parasite associated with damaged gill tissue (Figure 1.1C) [16, 17]. Histologically, the disease is characterized by lamellar fusion, epithelial desquamation, oedema, epithelial hyperplasia and interlamellar vesicle formation [17, 18]. An infiltration of leucocytes into the central venous sinus is also observed adjacent to infected regions and often increases with disease progression [18].



*obtained from CSIRO database

Figure 1.1 Gross pathology and histopathology associated with Atlantic salmon AGD. (A) Visual assessment of gross gill pathology in AGD-affected fish. (B) Fixed gill arch showing AGD-like white mucoid patches. **(**C) AGD lesion showing fusion of several secondary lamellae.

Clinical signs of AGD include lethargy, respiratory distress, lost of equilibrium and, if affected fish remain untreated, mortality [10, 19]. Typical signs of morbidity include a rapid decrease in feeding response and fish exhibiting characteristic gasping and 'coughing' opercular movements, before rolling over and sinking to the base of the net [20].

1.2. AGD treatment and monitoring

AGD has become a significant problem for Atlantic salmon growers worldwide. Nevertheless, despite years of research, the control of AGD still

restricted to bathing infected fish in freshwater [21], as the osmotic effect of bathing not only removes gill mucus and gill-associated amoeba, but also promotes a rapid healing of gill lesions [2, 22].

Freshwater bathing involves transferring the fish from the holding pen to a pen in which a canvas liner has been filled with freshwater. Fish are held at stocking densities of approximately 35–60 $kg.m^{-3}$, at oxygen saturations often from 100–200%, for a period of up to 2 h. Following bathing, the liner is pulled away and the fish are released into the underlying cage [23].

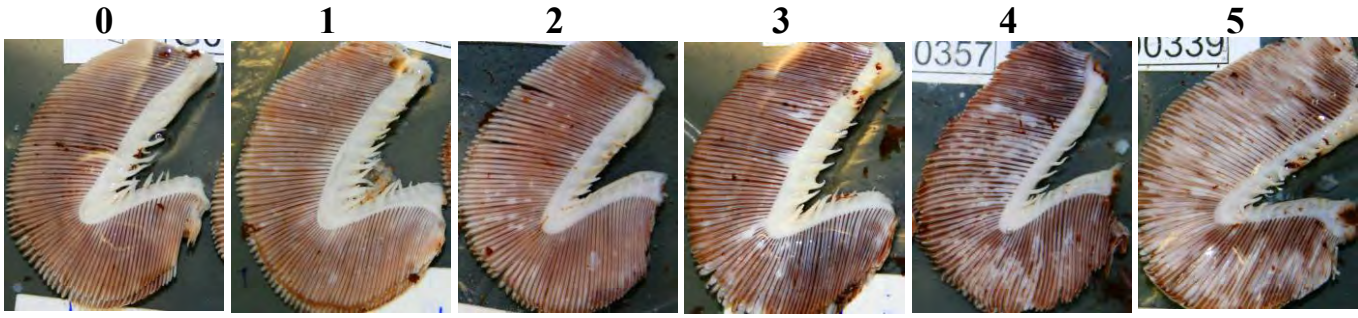
On-farm monitoring of AGD is regularly performed by assessing the gross gill pathology of a sub-population of fish from each pen [24]. The commercial producers utilize a categorical field evaluation of "gill score", that describes the extent of visible white patches on a scale of "clear" to "heavy" to schedule proactive freshwater bath treatments (Table 1.1) [25].

Table 1.1 Gross gill score system used by farmers to assess the severity of infection

Infection level	Gill score	Gross description
clear	0	no sign of infection and healthy red colour
very light	1	1 white spot, light scarring or undefined necrotic streaking
light	2	2–3 spots/small mucus patch
moderate	3	established thickened mucus patch / spot groupings up to 20% of gill area
advanced	4	established lesions covering up to 50% of gill area
heavy	5	extensive lesions covering most of the gill surface

*Reproduced from [25]

Baths are administered when the average gill score for the pen approaches a light to moderate level. The "gill score" is a gross measure of the degree of host response to the presence of amoeba (Figure 1.2). However, the degree of lesion development is known to be in direct proportion to the infective parasite concentration and progression of the infection [26].



*obtained from CSIRO database

Figure 1.2 Gill arches from AGD-infected Atlantic salmon and the corresponding indicative gill score.

1.3. AGD vs. Tasmanian Atlantic salmon industry

AGD has been considered the most serious disease facing the Tasmanian salmon industry for the past thirty years [27]. Despite the effectiveness of freshwater bathing, studies have shown that the numbers of gill-associated amoeba return to pre-bath levels within 10 days [22]. Re-infection is primarily due to waterborne trophozoites attaching to healthy gill tissue, but may also occur from low numbers of amoebae remaining upon the gills post bathing [18]. Pre-existing proliferative epithelial tissue appears to have an inhibitory effect upon trophozoite attachment [28], but these lesions heal rapidly and are then available for re-infection.

Since the time when AGD outbreaks were first reported in Tasmania, an increased frequency of freshwater bathing has been required during the summer months, and the need for freshwater bathing has been extended through most of the year [21]. According to Taylor et al. [20], each pen of fish is bathed 8-12 times throughout the 15-18 month production cycle, using approximately 500 L of freshwater per fish. As a result, managing AGD is estimated to cost the industry AU$ 40-50 million a year in treatment and lost productivity, as freshwater affects fish growth, as well as requires significant infrastructure and labour resources [8].

Another further limiting factor associated with this practice is the availability of freshwater in Tasmania, which can be in short supply during dry summers.

1.4. Attempts to control AGD

Due to the freshwater limitation in Tasmania and the high financial and logistical costs associated with freshwater bathing, such practice is not considered a viable long-term management solution. Consequently, the development of improved strategies for coping with AGD is required. In this context, a number of alternative treatments have been investigated with some success, including levamisole [29], chloramine-T [30], chlorine dioxide, hydrogen peroxide [31] and bithionol [32]. Similarly, the use of immunostimulants such as CpG oligodeoxynucleotides have also shown some promise [33]. However, much of the research into ameliorating the impact of the disease is focussed on the development of an effective vaccine, as it would improve fish welfare through the summer months, reduce or eliminate the need for freshwater bathing and potentially improve salmon growth rates, making Tasmanian salmon more cost-competitive in the global market.

Previous efforts to vaccinate against AGD have resulted in no increase in protection despite the presence of anti-*Neoparamoeba* antibodies in the sera [15, 34]. Active immunization has been attempted using intraperitoneal injection or anal intubation of both live and sonicated wild-type or cultured *Neoparamoeba*, either in the presence or absence of adjuvants [15, 34]. Bath exposure to either cultured or infective wild type antigens has also been attempted, but failed to provide protection [35]. In some instances, the results could be due to the use of the non-infective strains, as studies have demonstrated that traditional culture methods select for non-

virulent species of *Neoparamoeba*, even when the virulent species is present in the inoculating material [13].

The inability to culture the infective strain from the *Neoparamoeba* genus has significantly hampered the progression of research towards the development of alternative treatments against AGD, as experimental infections could only be established by cohabitation with infected fish [2] or by exposure to parasites freshly isolated from infected fish [26]. However, a recent study performed by Crosbie et al. [36] demonstrated a successful *in vitro* culture method for *N. perurans*, in which continued virulence was retained for at least 70 days.

The development of an *in vitro* system for AGD will certainly open new doors in the search of new treatment technologies. Therefore, unravelling the complexity of the parasite biology, as well as the intricate relationship with their host is imperative for the identification of new target candidates against AGD. In this context, RNA interference (RNAi), an efficient and highly specific gene silencing tool, could be employed not only to generate information about the largely uncharacterized gene functions in *Neoparamoeba* genus, but also to study the parasite - host interface through the identification of pathogenic determinants.

1.5. Using RNAi to study gene function and validate new drug targets against AGD

RNA interference (RNAi) is an evolutionarily conserved mechanism of sequence-specific gene silencing that reduces the levels of protein products translated from a targeted mRNA [37]. In short, the pathway is triggered by long double-stranded RNAs (dsRNAs) which are processed by the endoribonuclease Dicer into small interfering RNA (siRNA) oligonucleotides. The siRNA is then incorporated

into the RNA-induced silencing complex (RISC) and used as a template for sequence-specific degradation of the complementary mRNA [38]. A more detailed description of the RNAi pathway is found on Chapter 2.

The ability of RNAi to reduce the levels of specific proteins has had a profound impact on the study of gene function *in vitro*, especially in species that are not amenable to classical genetic approaches. The multi-step processes of RNAi can be experimentally activated at different stages by specific forms of regulatory RNAs. These include *in vitro* transcribed [39] and bacterially expressed dsRNA [40], *in vitro* [41] and chemically synthesised siRNA [42], Dicer -generated siRNA pool (d-siRNA) [43], viral/plasmid-based hairpin RNAs (shRNAs) [44, 45], miRNA [46, 47] and pre-miRNA [48]. Among the delivery methods that have been developed for efficient administration of these classes of nucleic acid, both *in vivo* and *in vitro*, are ingestion [49], injection [50], immersion [51], transfection [52] and electroporation [47].

With regards to AGD, RNAi could become an invaluable research tool to unravel the role of proteins essentially involved in amoeba attachment and pathogenicity, as well as to validate important targets by investigating the knockdown effect on amoeba viability, growth and replication. Additionally, RNAi technology could greatly assist with the elucidation of possible factors associated with the loss of virulence in certain *Neoparamoeba* strains. However, before RNAi-based approaches find its use against AGD, it is important to consider whether the causative agent of the disease possesses the required set of proteins involved in the RNAi pathway.

Although widely conserved in a variety of eukaryotic systems, many studies have shown that the RNAi machinery appears to have been repeatedly lost in certain protozoan parasites during evolution [53]. Surprisingly, the presence of functional

RNAi machinery has not shown to be conserved even amongst members of the same family [53, 54]. For example, genomic and functional studies previously demonstrated that within trypanosomatid protozoans RNAi was absent in both *Leishmania major* and *Trypanosoma cruzi* [55, 56]. On the contrary, a functional RNAi pathway and associated genes are found in other members of the family, such as *T. brucei*, *T. congolense* and *L. braziliensis* [53, 57-60]. However, considering the major role that RNAi plays in metazoan gene regulation, the mechanisms leading to the disappearance of functional RNAi in certain protozoan parasites are still unknown.

Despite the absence of functional RNAi machinery in some protozoan species, bioinformatics and functional experimental strategies have shown that all the species of amoeba investigated up to date were found to be RNAi positive (Table 1.2). Such findings are a promising step forward towards the application of RNAi-approaches in *Neoparamoeba* spp.

Table 1.2: RNAi-associated genes and functional studies undertaken in amoeba species

Amoeba species	Dicer*	Argonaute-like*	Functional studies
Entamoeba histolytica	EAL45114	XP_656514.1 XP_656436.1 ENY64486.1	[61] [62] [63]
Acanthamoeba castellanii	XP_004368077.1 ELR15123.1	XP_004353057.1	[64] [65] [66]
Naegleria gruberi	EFC46563.1 EFC50206.1 EFC44666.1	EFC47389.1 EFC41986.1 EFC49085.1	[67] [68]
Dictyostelium discoideum		XP_645445.1 XP_643218.1 XP_001134555.1 XP_636288.1 XP_635708.1 EAL71514.1	[69] [70] [71]

*accession number

1.6. Aim of the study and thesis outline

The main purpose of the present study was to use functional and comparative genomics approaches to investigate whether the RNAi mechanism has been retained or lost in species from the *Neoparamoeba* genus. As the *in vitro* culture of *N. perurans* has only been achieved recently [36], most of the experiments were performed using *N. pemaquidensis*.

The present thesis is presented in form of manuscripts. Therefore, part of the content found in the introduction and methodology of each chapter may be addressed more than once throughout the dissertation. The manuscripts have been either published (Chapter 2) or submitted (Chapter 3, 4 and 5) to peered-reviewed journals.

Chapter 1 briefly outlines the background and statement of the problem, as well as the purpose and significance of the study. The following chapter will examine and discuss the current literature regarding the application of RNAi technology as a therapeutic strategy for controlling disease in aquaculture. The content of Chapter 2 has been published as a review paper in *Fish & Shellfish Immunology*. In chapter 3 a *N. pemaquidensis* transcriptome database was utilized to identify transcripts encoding putative proteins associated with the RNAi pathway. Candidate proteins containing conserved domains found in Dicer and Argonaute were identified and their involvement in RNAi mechanisms validated following the administration of *in vitro* transcribed dsRNA and endoribonuclease-generated siRNA pool (esiRNA), respectively. Functional evidence for the presence of active RNAi machinery was also supported by RNAse III assay and esiRNA-mediated gene silencing. Further indication of functional RNAi pathway in *N. pemaquidensis* is demonstrated in the following two chapters, where successful knockdown of *N. pemaquidensis* target genes was achieved by the administration of bacterially

expressed dsRNA, either by soaking (Chapter 4) or feeding (Chapter 5). Chapter 6 validates the development of a successful *in vitro* culture of *N. perurans* and addresses the current status of RNAi-based approach in this species. Finally, chapter 7 draws together the research components to conclude the thesis and to raise future directions in the application of RNAi against AGD.

CHAPTER 2: Literature Review

Exploring RNAi as a therapeutic strategy for controlling disease in aquaculture

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Abstract

Aquatic animal diseases are one of the most significant constraints to the development and management of aquaculture worldwide. As a result, measures to combat diseases of fish and shellfish have assumed a high priority in many aquaculture-producing countries. RNA interference (RNAi), a natural mechanism for post-transcriptional silencing of homologous genes by double-stranded RNA (dsRNA), has emerged as a powerful tool not only to investigate the function of specific genes, but also to suppress infection or replication of many pathogens that cause severe economic losses in aquaculture. However, despite the enormous potential as a novel therapeutical approach, many obstacles must still be overcome before RNAi therapy finds practical application in aquaculture, largely due to the potential for off-target effects and the difficulties in providing safe and effective delivery of RNAi molecules *in vivo*. In the present review, we discuss the current knowledge of RNAi as an experimental tool, as well as the concerns and challenges ahead for the application of such technology to combat infectious disease of farmed aquatic animals.

Keywords: RNA interference; aquaculture; gene function; infectious diseases

2.1. Introduction

Aquaculture has expanded rapidly to become a major economic and food-producing sector in the world [72]. In parallel, due to the intensification of rearing methods and systems, the industry has been overwhelmed with a number of trans-boundary aquatic animal diseases caused by viruses, bacteria, fungi and parasites, with newer pathogens being identified every year [73]. Consequently, disease outbreaks have become a significant constraint to the development of the aquaculture industry, affecting the socio-economic development of this sector worldwide [74]. Therefore, in order to meet the increased demands of our expanding population, new technologies and techniques for disease control must be developed and implemented. Of major interest is the application of genetic engineering and other biotechnologies.

One recent technology likely to play a major role in the future of aquaculture is RNA interference (RNAi). RNAi is a recently-discovered mechanism of post-transcriptional gene silencing in which double-stranded RNA (dsRNA) corresponding to a gene, or coding region, of interest is introduced into an organism, resulting in degradation of the corresponding mRNA [75]. Because of this sequence-specific ability to silence target genes, RNAi has been extensively used to investigate the functional role of specific genes by reducing expression, without altering genotypes [76]. The silencing effects could be used not only to study gene function, but also to indentify drug targets and vaccine candidates [77], as well as to control infectious disease by interfering with pathogen transmission, development and proliferation within the host [76].

In this review we summarize the current knowledge regarding the therapeutic applications of RNAi for developing alternative treatment strategies against infectious diseases in aquaculture.

2.2. RNA interference

RNAi is a highly evolutionally conserved process of post-transcriptional gene silencing (PTGS) by which dsRNA, when introduced into a cell, causes sequence-specific degradation of homologous mRNA sequences [78]. RNAi as a mechanism of PTGS most likely evolved as a cellular defence strategy to eliminate unwanted nucleic acids (viruses and transposable elements) in plants, fungus and invertebrates [79], but is also widely employed in most eukaryotic cells as a mechanism to regulate the expression of endogenous genes [80].

The discovery of RNAi phenomenon was first observed when plant biologists were performing experiments to enhance the hue of purple petunias. The introduction of a pigment-producing gene under the control of a promoter resulted in variegated or completely white flowers, rather than the expected deep purple colour [81]. What was initially thought to be a peculiar effect in flowers was subsequently found to occur in fungi when scientists were attempting to boost the synthesis of orange pigment in *Neurospora crassa* [82]**.** The phenomenon was first called co-suppression in plants and quelling in fungi. The observation of RNAi in animals came accidently when Guo and Kemphues [83] injected the anti-sense strand to block expression of the par-1 gene in the nematode *Caenorhabditis elegans*. The expression was disrupted but, upon performing their controls, they found that the sense strand also reduced the expression of that gene. The involvement of dsRNA in gene silencing phenomena, however, was discovered by Fire et al. [84] who found that dsRNA, but not single stranded sense or antisense RNA, mediated gene silencing in microinjected *C. elegans*. Subsequently, RNAi has been recognized as a highly conserved process encountered not just in unicellular protozoans and fungi but also in complex organisms such as plants and animals [57, 85-88].

The basic principle of RNAi involves destruction of mRNA upon interaction with homologous dsRNA, and translational repression through imperfect complementary binding of small RNAs with the 3′ untranslated region of the target mRNA [89]. Genetic and biochemical data indicate a possible two-step mechanism for RNAi: an initiation step and an effector step [75] (Figure 2.1).

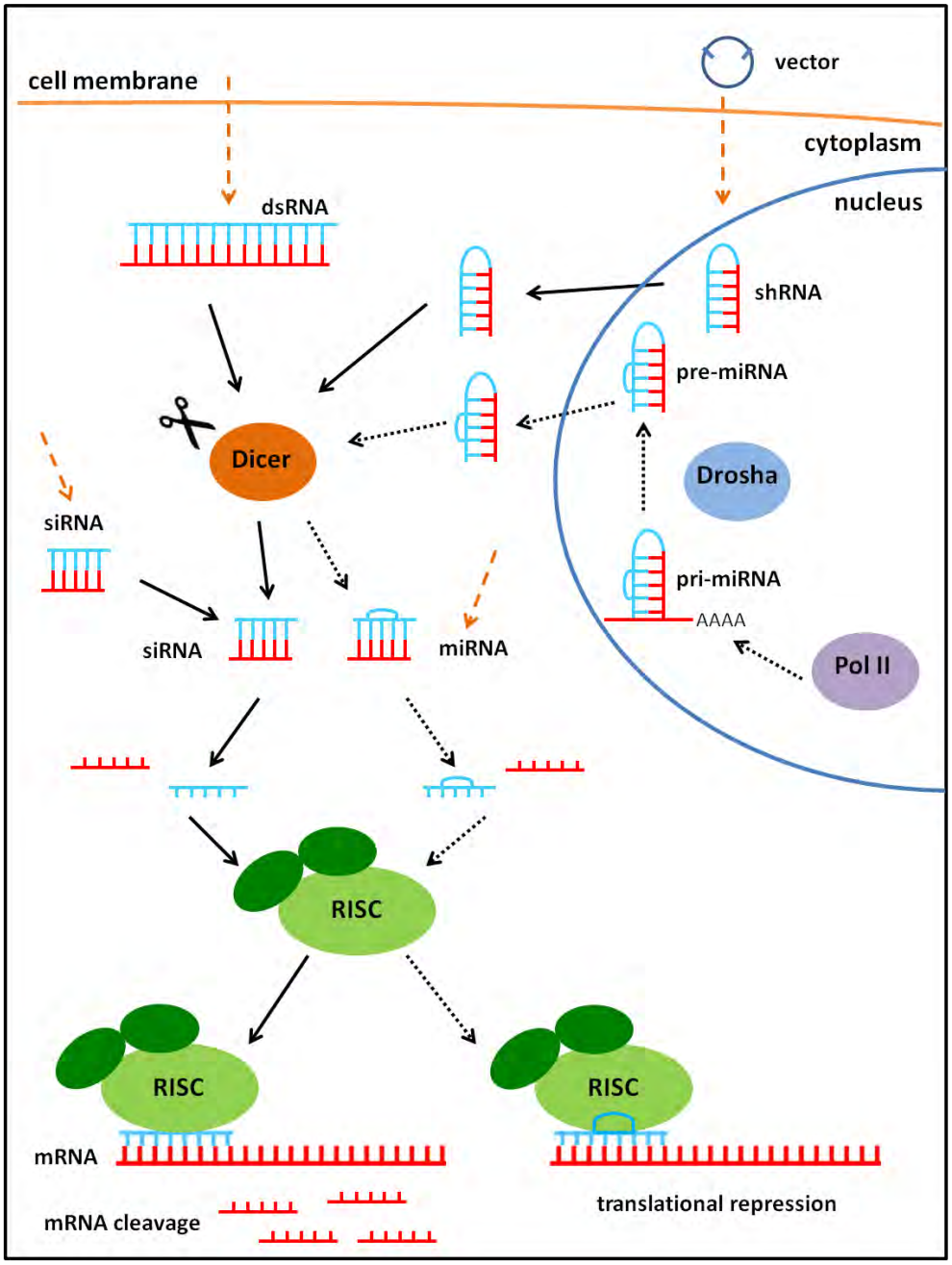

Figure 2.1 A simplified model for the endogenous RNAi pathway. Solid and dotted arrows represent the siRNA and miRNA pathways, respectively. Orange dashed arrows indicate possible exogenous RNAi triggers, such as synthetic dsRNAs, siRNAs and miRNA, as well as vector-based shRNAs.

In the initiation step, long dsRNA, derived from endogenous, transgenic or viral transcripts, are processed into short ~ 21–22 bp molecules, known as small-interfering RNA (siRNA). Those small molecules have a characteristic 2 nucleotide 3′ overhang that allows them to be recognized by the enzymatic machinery of RNAi that eventually leads to homology dependent degradation of the target mRNA [90]. The generation of siRNA during the initial steps of RNAi is performed by a Ribonuclease III (RNase III) enzyme, called Dicer, which has the ability to recognize and cleave dsRNA at specific positions or sequences [91]. During the effector step, the short RNA duplexes are incorporated into a multimeric protein complex, known as RNA-induced silencing complex (RISC), which contains an Argonaute (Ago) protein as one of its main components. RISC binds and unwinds the siRNAs into single-stranded molecules. The sense strand is released and the antisense strand remains bound to RISC, serving as guide to select fully complementary mRNA substrates for degradation [92].

Besides siRNA, another class of small RNAs, known as microRNAs (miRNAs), have been characterized, sharing a common RNase-III processing enzyme (Dicer) and closely related effector complex (RISC), for post-transcriptional repression. However, while siRNAs originate from exogenous DNA or dsRNA, aberrant transcripts from repetitive sequences in the genome or long hairpin forming transcripts, miRNAs are derived from precursor transcripts called primary miRNAs (pri-miRNAs), which are typically transcribed by RNA polymerase II [93]. Pri-miRNAs are cleaved in the nucleus by a Dicer-like enzyme called Drosha to produce a characteristic stem-loop structure of about 70 bp long, known as pre-miRNA [94]. Subsequently, pre-miRNA is transported to the cytoplasm, where the loop end is cleaved off by Dicer activity, resulting in a mature miRNA, a dsRNA approximately 22 bp in length [95]. The mature miRNA then enters RISC and exert its regulatory

effect via either degradation of complementary mRNA or binding to imperfect complementary sites within the 3' untranslated region (UTRs) of their mRNA targets, leading to translational repression [96]. The mature miRNA then enters RISC, which recognizes target mRNAs based on sequence complementarity between the guide miRNA and the mRNA transcript resulting in either mRNA degradation or translational repression [96] (Figure 2.1).

Apart from PTGS, another homology-dependent gene silencing mechanism called transcriptional gene silencing (TGS) is recognized in plants and animals [97]. TGS is induced by the same molecules that trigger PTGS, but results in inactivation of the gene for transcription rather than by RNA destruction [98]. TGS inhibits transcription by DNA methylation and histone post-translational modifications, and corresponding remodelling of chromatin around the target gene into a heterochromatic state [99]. As TGS is not the focus of the present, more detailed information can be found in other reviews [97, 100, 101].

The multi-step processes of RNAi can be experimentally activated at different stages by specific forms of regulatory RNAs. These include *in vitro* transcribed [39]and bacterially expressed dsRNA [40], *in vitro* [41] and chemically synthesised siRNA [42], Dicer -generated siRNA pool (d-siRNA) [43], viral/plasmid-based hairpin RNAs (shRNAs) [44, 45], miRNA [46, 47] and pre-miRNA [48]. Among the delivery methods that have been developed for efficient administration of these classes of nucleic acid, both *in vivo* and *in vitro*, are ingestion [49], injection [50], immersion [51], transfection [52] and electroporation [47].

2.3. RNAi as a natural antiviral defence mechanism in vertebrates and invertebrates

To protect themselves from viral infections, most organisms have evolved several defence mechanisms to sense and fight those pathogens. Among those systems are the interferon (IFN)-mediated [102] and the ancient recently described RNAi-based antiviral mechanism [103]. In both systems, dsRNAs present in the viral genome or commonly generated during viral replication are recognized by the host as molecular pattern associated with viral infection, inducing a range of immune responses [79].

The post-transcriptional activity of the RNAi machinery to degrade cytoplasmic RNA in a sequence specific manner is the key to its antiviral function in invertebrates [104]. In this process, virus-derived siRNAs originated by Dicer cleavage are incorporated into the RISC, leading to the degradation of the corresponding viral RNAs and, consequently, inhibition of viral replication [105].

In vertebrate cells, on the other hand, the introduction of long dsRNA typically induces innate immune responses, constituting the first line of defence to limit viral replication [106]. The intracellular presence of viral dsRNA activates the Toll-like receptor (TLR) 3 pathway, as well as dsRNA recognition proteins (dsRNA-dependent protein kinase PKR and 2′-5′-oligoadenylates/RNase L), leading to the nonspecific degradation of RNA transcripts, the production of IFN response and the overall shutdown of host cell protein syntheses [107]. The nonspecific inhibitory effects caused by activating the IFN pathway initially hampered the application of RNAi in vertebrate cells. This problem was later circumvented by Elbashir et al. [108], who showed that siRNAs (roughly 20-25 bp), rather than long dsRNA (>30 bp), effectively knock-down the amount of transcript of a given gene without

activating the IFN system. However, the minimum effective siRNA dose is often recommended to be employed, as high siRNA concentrations have also proven to activate components of the IFN system [109-112]. Such discovery ignited an explosion of work on RNAi in vertebrate cells. Nevertheless, it is still unclear whether RNAi naturally acts as a system of defence against viral infection in vertebrates.

Given the potential of the innate immune response to promote an effective antiviral response based on protein recognition, it is expected that in vertebrates the RNAi machinery has been conserved for other purposes, rather than an ancient nucleic-based antiviral response [113]. However, an increasing number of studies have shown evidence that RNAi-mediated silencing mechanism is is an important component of the mammalian antiviral response [114-116]. While invertebrates produce virus-derived siRNAs to direct antiviral immunity, Pfeffer et al. [117] demonstrated that infection of mammals with certain DNA virus induces production of virus-derived miRNAs. The mechanisms involved in the interaction between viral and cellular miRNA in mammals are well covered in numerous reviews [118-121], suggesting that not only viral miRNAs can directly alter host physiology, including components of the immune system, but also that host miRNAs and other components of the miRNA pathway can interact with viruses at multiple levels to influence viral replication. The evidence presented above reinforces the potential of utilising virus-infection systems as models to refine our understanding on the molecular determinants of RNA silencing in vertebrate cells.

2.4. RNAi applied to aquaculture

Aquatic animal diseases are the most significant constraint to the development and management of aquaculture worldwide. As aquaculture is growing rapidly and poised to help in bridging the gap in the global supply and demand of aquatic animal food products, management of health and reducing losses due to disease in aquaculture is gaining high priority. A variety of technologies, in particular biotechnology, have already made an impact in reducing aquatic animal disease risk and many novel methods will contribute in the future [122].

Recent advances involving the use of RNAi-based technologies promise alternative approaches for the stable silencing of genes in a variety of different animal species [123]. Among its many applications, RNAi stands out as a powerful molecular tool to screen host genes involved in pathogenicity and other important biological processes, as well as to validate potential drug targets [124]. In addition, silencing of viral genes stands out as a promising therapeutic approach for the development of antiviral strategies in organisms that can mount systemic antiviral RNAi response. In this context, RNAi could help stop virus replication inside the host, reducing virus spread and, consequently, help the control of a possible outbreak of the disease [125]. With regard to parasitic infections, the analysis of gene function through RNAi could be used not only to investigate the interaction between host and parasite, but also to explore the parasite biology and the effects of knockdown on its survival. This could assist in the identification and validation of new anti-parasitic drug targets [76, 126].

2.4.1. RNAi as an antiviral tool in fish

Viruses are recognized as the most numerous organisms in the marine environment [127]. However, while viral diseases can cause problems among natural fish stocks, these infectious agents are devastating and costly in aquaculture where fish are confined and intensively reared [128].

Viruses of lower vertebrates include a large number of viral agents, belonging to different viral families and genera, with RNA and DNA genomes, displaying different host specificities [129]. The most important viral diseases affecting farmed fish worldwide are caused by different genera, mostly within the families: *Rhabdoviridae*, *Nodaviridae*, *Birnaviridae* and *Iridoviridae* [130].

Some of the most significant viral pathogens of fish are members of the family *Rhabdoviridae*, which are enveloped negative single stranded RNA viruses. The diseases caused by those viruses are generally characterized as acute, hemorrhagic septicaemias affecting multiple organs [131]. Two fish rhabdoviruses, *Infectious haematopoietic necrosis virus* (IHNV) and *Viral haemorrhagic septicaemia* (VHSV), have received special attention due to rapid progression of infection and high mortality, especially in farmed salmonids [132]. *Hirame rhabdovirus* (HIRRV) is also another economically significant *Rhabdovirus* known to cause epidemics in farmed fish [130].

Nodaviridae, on the other hand, are a family of small nonenveloped, isometric riboviruses, with bipartite positive-sense RNA genomes [133]. Piscine nodaviruses belong to the genus *Betanodavirus*, which are the causative agents of *Nervous necrosis virus* (NNV), a devastating neuropathological condition that causes high mortalities in a variety of cultured marine fish [134].

Members of the *Birnaviridae* have single-shelled nonenveloped capsids and genomes comprising two segments of double-stranded RNA [130]. This family is mostly represented by the *Infectious pancreatic necrosis virus* (IPNV), an acute contagious systemic disease that causes gastroenteritis and destruction of the pancreas in several species of freshwater and marine fish [135].

Iridoviruses are large double stranded DNA viruses with an icosahedral capsid ranging from 120 to 350 nm in diameter [130]. Members of the family *Iridoviridae* are an emerging group of viral pathogens that threaten the aquaculture industry, causing great economic losses throughout the world. *Rock bream* and *Red sea bream iridovirus* (RBIV and RSIV), as well as *Infectious spleen and kidney necrosis virus* (ISKNV) and *Rana grylio virus* (RGV), are some of the viral pathogens of fish caused by iridoviruses [136].

Considering the substantial economic, social and environmental impact of emerging viral diseases in aquaculture, a considerable amount of research has been undertaken on viruses that cause economically important diseases. However, despite the amount of investigation undertaken, there are few vaccines available for the prevention of many piscine infectious diseases, especially those of viral origin [137]. Therefore, a better understanding of viral replication mechanisms, as well as the determinants of virulence, is essential to assist on the development of effective prevention methods to inhibit virus replication in fish. Up to now, two review papers addressing the use of RNAi-mediated gene silencing in fish have been published [138, 139]. However, these mainly focus on studies performed in zebrafish embryo and cell lines, with only superficial coverage of the potential use of RNAi to combat pathogenic fish viruses. As the RNAi technology has been extensively employed to investigate the role of several viral related genes in the replication of viruses that

cause major economic losses in cultured fish worldwide (Table 2.1), this review will mainly focus on those studies.

2.4.1.1. RNAi-mediated viral immunity in fish

In recent years, RNAi mediated by siRNA has been shown to have activity against a wide range of viruses and is considered a potential antiviral tool, especially in organisms that have evolved the RNAi-mediated viral immunity [140]. It is of common knowledge that while the vertebrate animals rely on the IFN pathway as their primary innate immune response to virus infection, the invertebrates, which mostly appear to lack such system, depend on the antiviral RNAi defence system [102, 103]. However, indirect evidence for the persistence of RNAi-mediated antiviral immunity in fish have been shown in the literature [141-144].

Fenner et al. demonstrated that fish *Betanodavirus* possess a small protein named B2, which binds to and protects dsRNA from Dicer cleavage and subsequent suppression of the RNAi silencing pathway [141]. Using a mutant B2 designed to lack residues essential for dsRNA binding and protection, a further study performed by the same research group [142] showed that while wild B2 efficiently suppressed silencing of green fluorescent protein (GFP) gene, the mutant exhibited a significant reduction in its ability to block gene knockdown. Additionally, it was shown that siRNA-mediated downregulation of Dicer significantly increased the accumulation of mutant B2 in HeLa cells. The results altogether provide strong evidence that fish *Betanodavirus* have evolved a strategy to sustain replication by blocking the RNAi pathway, suggesting that such mechanism also acts as an antiviral immune system in fish.

Table 2.1 Summary of RNAi studies applied to fish viruses

Ref	Cell type	Target gene	Delivery method	Pathogen	RNAi inducer
[89]	rainbow trout embryous	GFP and tyrosine A	microinjection	-	chemically synthesized siRNA
[34]	FHM cells	MCP and lacZ reporter	transfection	TFV	*in vitro* transcribed siRNA/vector-based shRNA
[94]	chinnook salmon embryo	nucleoprotein-N	transfection	VHSV	DsiRNA
[37]	EPC cells	L-polymerase	transfection	VHSV	vector-based siRNA
[90]	EPC cells	glycoprotein	transfection	VHSV	*in vitro* transcribed siRNA
[38]	EPC cells	glycoprotein	transfection	VHSV	vector-based shRNA
[93]	EPF and CHSE-214 cells	glycoprotein	transfection	VHSV	vector-based dsRNA
[43]	juvenile rainbow trout	glycoprotein	IP injection	VHSV	chemically synthesized siRNA
[98]	rainbow trout	GFP and GAPDH	IP injection	VHSV	chemically synthesized siRNA
[41]	HINAE cells	MCP and glycoprotein	transfection	RSIV-HIRRV	virus-encoded pre-miRNA
[35]	HINAE cells	MCP	transfection	RSIV	vector-based siRNA
[92]	GF cells	MCP	transfection	RBIV	vector-based siRNA
[97]	juvenile rock bream	MCP	muscular injection	RBIV	in vitro transcribed dsRNA
[91]	CIK cells	RdRp and OCP	transfection	GCRV	chemically synthesized siRNA
[45]	CIK cellc	RdRp and OCP	transfection	GCRV	plasmid-transcribed shRNA
[39]	GCO cells	viral envelope protein	transfection	RGV	amiRNA
[100]	CHSE-214 cells	annexin 1	transfection	IPNV	chemically synthesized siRNA
[99]	cBB cells	Mx protein	transfection	NNV	chemically synthesized siRNA
[101]	pufferfish	DIGIRR	muscular injection	-	lentivirus-based siRNA

amiRNA, artificial micro RNA; cBB, barramundi brain cells; CHSE-214, chinook salmon embryos; CIK, grass carp kidney cells; DIGIRR, Ig IL-1R related molecule; DsiRNA; EPC, epithelioma *Papulosum cyprini* carp cells; FHM, fathead minnow cells; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCO, grass carp ovary cells; GCRV, *Hemorrhagic Virus of Grass Carp*; GF, grunt fin cells; GFP, green fluorescent protein; HINAE, hirame natural embryo cells; HIRRV, *Hirame Rhabdovirus*; IP, intraperitoneal; IPNV, *Infectious Pancreatic Virus*; MCP, major capsid protein; NNV, *Nervous Necrosis Virus*; OCP, outer capsid protein; RBIV, *Rock bream Iridovirus*; RdRp, RNA dependent RNA polymerase; RGV, *Rana grylio* Virus; RSIV, *Red Sea Bream Iridovirus*;; TFV, Tiger Frog Virus; VHSV, *Viral Hemorrhagic Septicemia Virus*

Another clue for the presence RNAi-mediated antiviral immunity in fish was proposed by Su et al. [143] when investigating the role of rare minnow, *Gobiocypris rarus*, Dicer during infection with *Grass carp reovirus* (GCRV). The results showed that Dicer was significantly up-regulated in the liver during the first 24 h post viral injection, suggesting that viral- dsRNA can activate the RNAi-machinery in fish. The identification of systemic RNAi defective protein 1 (SID-1) in mandarin fish *Siniperca chuatsi* (ScSidT2), a protein known to play an important role in cellular dsRNA uptake, also supports the involvement of RNAi in fish antiviral defence [144]. Using an *in vitro* assay, the authors demonstrated that while *Sc*SidT2 mRNA levels significantly increased following challenge with ISKNV, viral replication remarkably increased when the function of *Sc*SidT2 protein was blocked. All the unpredicted findings commented above bring hope to the industry, as RNAi technology might find a use on the development of antiviral strategies in fish.

2.4.1.2. Gene silencing mediated by in vitro transcribed and chemically synthesized siRNAs

The first evidence that siRNA-mediated gene silencing is effective in fish was demonstrated by Boonanuntanasarn et al. [145], using transgenic rainbow trout embryos carrying the GFP gene. According to the results, siRNA was able to interfere with transient and stable transgene expression during embryo development, as well as inhibit the expression of maternally inherit mRNA. The feasibility of RNAi technology as a tool to control fish viral diseases, on the other hand, was initially reported by Xie et al. [41]. For this purpose, *in vitro* transcribed siRNA was used to silence the expression of the major capsid protein (MCP) encoded by tiger frog virus (TFV), a member of the *Iridoviridae* family. The results provided evidence

that siRNA effectively inhibited TFV replication in fathead minnow (FHM) cell lines. However, despite using mismatched controls to check for specificity of their siRNAs, the authors did not verify if virus replication was inhibited non-specifically via IFN response. The T7 phage polymerase transcribed siRNAs have been shown to trigger IFN responses in mammalian cell lines [109]. Therefore, due to its antiviral nature, a cellular IFN response should be considered in studies using *in vitro* transcribed siRNA, where a reduction in viral replication is frequently interpreted as a successful specific interference. This assumption was corroborated by Schyth et al. [146], using a heterologous virus as a control for target specificity. According to their results, *in vitro* transcribed siRNAs specific to the VHSV glycoprotein (VHSV-G) efficiently inhibited viral multiplication in cell cultures infected with the target virus, suggesting specific interference. However, the transcribed siRNAs similarly protected the cells against the control virus, SVCV. Further analysis revealed that activation of the IFN system, induced by siRNAs, was responsible for the nonspecific viral inhibition.

Dang et al. [42] described the antiviral activity of chemically synthesized siRNAs designed to target the MCP gene of RSIV. HINAE cells transfected with the MCP-expressing plasmid (pCMV-MCP) showed inhibition of RSIV replication by reduction of both MCP expression levels and viral production. The specificity of the interference was analysed by using mismatched siRNA controls, as well as an identical sequence specific for MCP of TFV. However, possible immunostimulatory side effects of synthetic siRNAs were not taken in account by the authors. No IFN response was also verified by Li et al. [147] when investigating the supposed viral inhibitory effects of chemically synthesized siRNA designed against the GCRV RNA dependent RNA polymerase (RdRp) and outer capsid protein (OCP) genes. Similarly, neither the activation of a possible shRNA-mediated IFN response, nor the

use of heterologous virus and mismatched shRNA controls were considered by Ma et. al [52], when exploring the antiviral effect of plasmid-transcribed shRNAs synthesized against the same GCRV genes. Therefore, the above results should be interpreted with caution as there is not convincing evidence that the observed therapeutic effects were caused by target-specific RNAi-mediated gene silencing rather than nonspecific stimulation of the innate immune system.

2.4.1.3. Gene silencing mediated by vector-based RNAi constructs

Aiming to increase the amount of antiviral siRNAs per cell, a different strategy than transfection was reported by Ruiz et al.[44]. A fish cell line which constitutively expresses siRNA was developed by transforming epithelioma papulosum cyprinid (EPC) cells with cytomegalovirus (CMV) promoter-driven siRNA expression vector, which was designed against VHSV-L polymerase gene. According to the results, sequence-specific interference was detected when EPC clones were transformed with a mixture of three shRNAs, but not with individual shRNAs. However, like in previous studies, the interference was not specific for VHSV, as infection with a heterologous virus (IHNV) was also reduced to a similar extent. On the other hand, no significant up-regulation of carp Mx1 was detected, suggesting the antiviral activity was caused by an unknown nonspecific inhibitory mechanism, unrelated to the IFN response. A possible interaction of the shRNAs with the viral double-stranded replicative intermediate might explain the lack of specificity reported by the authors.

Similar results were also reported by Kim and Kim [45] when using a fugu-U6-promoter-driven shRNA designed to target the VHSV-G gene, to investigate inhibition of VHSV replication, in EPC cells. However, in contrast to what was

found by Ruiz et al. [44], the authors demonstrated that endogenous shRNA not only effectively knocked down the target gene, but also provided a sequence-specific RNAi-dependent suppression, as protection was not conferred against the control virus (IHNV). The RNAi mediated inhibition was confirmed by gene expression analysis, where no IFN-related response was detected in transfected cells. A more stable and efficient siRNA expression vector utilizing a modified rock bream β-actin promoter was also developed by the same research group [148]. However, expression analysis of viral mRNA was reduced only up to 48 h post viral inoculation, suggesting the inhibitory effect of siRNA expressed from plasmid vector might not be long lasting. The most recent attempt by the same group was to construct a long dsRNA producing vector driven by fugu double U6 promoters [149]. Surprisingly, the results showed for the first time that plasmid-based long dsRNA targeting the VHSV-G gene successfully inhibited viral proliferation in a sequence-specific manner, without triggering IFN response and cross-protection against the control virus (IHNV). This new system could simplify RNAi-screening by avoiding the need to design and select of effective siRNAs. However, it should be taken into consideration that siRNAs generated from long dsRNA can induce specific off-target effect by interfering with the expression levels of non-target genes.

2.4.1.4. Gene silencing mediated by other RNAi triggers

No evidence of IFN activation was also found by Kim et al. [46], when using envelope protein (53R) targeted artificial microRNAs (amiRNAs) for silencing the iridovirus *Rana grylio* virus (RGV) *in vitro*. The results demonstrated that amiRNA-R53 successfully silenced the expression of the target gene, affecting not only the virions assembly and replication, but also reducing RGV titer and delaying the

emergence of cytopathic effect (CPE). However, despite the silencing specificity, the inhibition efficiency achieved by the amiRNA construct was considerably low (58%). Dang et al. [48], in contrast, showed that engineered virus-encoded pre-microRNAs (pre-miRNAs) designed to express miRNAs specific to HIRRV genome and to MCP gene of RSIV, induced potent antiviral responses in fish cells not only by miRNA-related pathways, but also by induction of IFN-related responses.

Recently, double stranded RNA Dicer substrate (DsiRNAs) designed for the generation of siRNA targeting the nucleoprotein N gene of VHSV (VHSV-N), was used to evaluate antiviral effect in fish cell lines [150]. Following VHSV challenge, a significant reduction of VHSV-N levels was observed in cell cultures transfected with the DsiRNAs targeting VHSV, in comparison with the control group, DsiRNAs targeting human influenza nucleocapsid. However, no significant differences in viral replication were detected between the treatments, when cells were infected with the closely related heterologous virus, IHNV. Besides demonstrating a highly specific reduction of viral replication, the authors also showed that the efficiency of VHSV-N mRNA knockdown was dependent on both the DsiRNA concentration and the dilution of the viral stock inoculations.

2.4.1.5. In vivo delivery of RNAi into fish

Activation of the IFN system was also reported *in vivo* by Schyth et al. [50], when evaluating the antiviral effects of intraperitoneally injected synthetic siRNA in juvenile rainbow trout, challenged with VHSV. While fish injected with naked siRNA targeting the VHSV-G (siVHSV-G) exhibited mortality curves identical to the control group, injection of polycationic liposome-formulated siRNA resulted only in a significant delay in mortality. The specificity of the interference was

investigated by the authors, as previous studies had shown an up-regulation of IFN genes in cells transfected with liposome-formulated polycationic transfection agent 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), *in vitro* [151] and *in vivo* [152]. For this purpose, DOTAP alone and DOTAP-formulated control siRNA targeting the reporter gene EGFP (siEGFP) were used under the same conditions described above. According to the results, a comparable protection was verified for siEGFP and siVHSV-G, while no effect was detected for DOTAP itself, suggesting a nonspecific antiviral protection induced by DOTAP/siRNA complexes. Those results were supported by the gene expression analysis, where an up-regulation of IFN was verified in the liver, indicating a correlation between protection and systemic IFN response.

The effect of long dsRNAs administered by intramuscular injection followed by rock bream iridovirus (RBIV) challenge, has also been tested [153]. While fingerlings injected with virus-specific dsRNA presented a delay on mortality in comparison to the PBS injected control group, no considerable differences in survival were detected in relation to the virus nonspecific GFP treatment. Once again, expression levels of IFN inducible MX gene indicated that a nonspecific type I IFN response, rather than a sequence-specific RNAi, was involved in protection.

Aiming to investigate whether chemical modification of siRNA duplexes have the ability to reduce the expression levels of genes involved in innate immune responses, Schyth et al. [154] recently developed an *in vivo* fish-interferon sensitive virus model that enables screening for desired siRNA modifications without compromising their knockdown efficiency. The results showed that some modifications distinctly affected the siRNAs antiviral effect, suggesting that optimization of modification type, number and location should be employed when

designing each target siRNA. This new model will allow researchers to further explore the involvement of siRNAs in nonspecific antiviral immunity *in vivo*.

Apart from the ability to induce antiviral innate immunity, RNAi has also been explored to investigate, *in vitro*, the role of genes involved in fish immune response, such as the involvement of barramundi Mx protein (BMx) in NNV replication [155] and the antiapoptotic function of salmon Annexin 1 [156]. The only functional study reporting *in vivo* administration of siRNAs into adult fish tissues was recently published by Gu et al. [157], to assist in the characterization of a novel double Ig IL-1R related molecule (DIGIRR). By using a lentivirus-based siRNA delivery system, the authors successfully delivered the DIGIRR-siRNA into the fish by muscle injection, effectively downregulating the target gene in immune-related tissues (liver and kidney) and cells (leucocytes). However, despite the results, further safety concerns, such as insertion mutagenesis and immunogenicity, as well as difficulties regarding large-scale manufacture [158] may limit the use of viral vectors for siRNA delivery in large scale.

2.4.1.6. The future of RNAi to control viral diseases in fish

Despite all the efforts that have gone into developing effective RNAi-based strategies in fish models over the last decade, the reality of commercially applying RNAi technology in aquaculture still distant. The studies undertaken to date show much inconsistency, especially when targeting genes from the same virus species, suggesting that the specificity of RNAi-mediated antiviral activity can be highly affected by many factors such as target gene, sequence, delivery system and dosage. The activation of IFN response is another significant hurdle that needs to be addressed before the whole promise of RNAi can be employed in aquaculture.

However, apart from all the issues regarding effectiveness of construct design and delivery strategy, finding a practical and cost effective delivery method in open water aquaculture systems will be the main drawback in the progress of large scale application of RNAi in fish farms. This issue will be further discussed latter in the review (section 2.5.2).

2.4.2. RNAi as an anti-parasitic tool in fish

The rapid expansion of cage culture for aquatic animals has been associated with the emergence of parasitic diseases [159]. Effective drugs for treatment are often available, but there are some limitations of their use due to parasite resistance, toxicity of chemicals and persistence of chemical residues [160]. Consequently, the negative impact of some parasites on fish health and its economic relevance in aquaculture and fisheries has enhanced the interest for improved methods of control [161], particularly the development of vaccines against the pathogens of most economic relevance [160].

Initially the success of antiviral [162, 163] and anti-bacterial [164] vaccines suggested that vaccine development for parasitic diseases would be straight forward. However, research towards parasite vaccines is still at an early stage, with no successful attempts, to date, in producing an effective commercially available vaccine against fish parasitoses [161]. The identification of good vaccine targets is one of the main obstacles hampering the progress of research in this field, partially due to the limited existing knowledge regarding the life-cycle and ecology of pathogenic parasites, as well as the immunological relationships between fish and their parasites [165].

RNAi has recently become a powerful tool to silence the expression of genes and analyse their loss-of-function phenotype. In this context, the function analysis of parasite genes, as well as the role of their products in parasite biology, shows strong potential in the identification and characterization of new vaccine candidates against fish parasitic diseases. However, despite being considered a useful and efficient tool for functional genomics, only a few studies have been undertaken regarding the use of RNAi to manipulate gene function of fish parasites.

The first successful *in vivo* demonstration of gene silencing mediated by dsRNA in a fish parasite was published by Ohashi et al. [51]. Aiming to establish a new control method of *Neobenedenia girellae*, a capsalid monogenean known to infect the body surface of many species of marine fish, the authors attempted to block or interfere with the gametogenesis, by focusing on three *vas*-related genes (*Ngvlg*1, *Ngvlg*2 and *Ngvlg*3). The results showed that introduction of dsRNA *Ngvlg*1 or *Ngvlg*2, by soaking, resulted in partial or complete loss of germ cells, as well as in a reduction of hatching rates, demonstrating that sterilized *N. girellae* could be successfully produced using RNAi.

Since the initial manuscript, the RNAi technology has been mostly applied to study gene function of sea lice (*Lepephtheirus salmonis* and *Caligus rogercresseyi*), which are copepod crustaceans responsible for one of the main disease outbreaks in salmon aquaculture. Dalvin et al. [166] established a dsRNA-mediated technique to assess the functional role of a *L. salmonis* yolk associated protein (*Ls*YAP). Immature females injected with dsRNA normally laid external egg strings, however the majority of the embryos were either unable to hatch, or produced strongly deformed larvae, demonstrating a crucial role for *Ls*YAP in the embryogenesis of salmon louse. On the other hand, Campbell et al. [167], analysed the feasibility of gene-knockdown in different life stages of *L. salmonis*, using a putative l

prostaglandin E synthase type-2 (PGES2) as a candidate gene. For this purpose, dsRNA was administered by injection or soaking, depending on the size of the parasitic stadia. Despite showing no significant effect on mortality rates, a downregulation of PGES2 was detected within 24h, regardless the delivery method or life stage analysed.

The most recent publication describing the use of RNAi in sea lice was published by Carpio et al. [165]. Degenerate primers based on arthropod sequences encoding akirin-2 proteins were used by the authors to isolate a novel gene from *C. rogercresseyi*. The new gene, called *my*32, was characterized and its function assessed by dsRNA mediated knockdown. The results showed a 70% downregulation of *my*32 transcripts in sea louse soaked with *my*32-dsRNA, compared to the control group. A considerable reduction in the number of ectoparasites was also observed in dsRNA treated fish, suggesting the *my*32 protein may be a promising target for vaccine development against sea lice infestations.

2.4.3. RNAi as an antiviral tool in shrimp

The control of viral diseases in shrimp remains a serious challenge for this important aquaculture industry, as outbreaks in economically important species, such as *Penaeus monodon* and *Litopenaeus vannamei*, has led to significant losses in production across different geographical locations, since the early 1990s [168].

More than 20 shrimp viruses have been identified. Among those, four are especially important due to their epizootic spread and economic impact: *White spot syndrome virus* (WSSV), *Yellow head virus* (YHV), *Taura syndrome virus* (TSV) and *Infectious hypodermal and hematopoietic necrosis virus* (IHHNV) [169, 170].

Besides viruses, shrimp are also susceptible to a wide variety of pathogens, including parasites, fungi, protozoa, rickettsia and bacteria [171]. However, while bacterial, fungal and protozoan caused diseases can be managed by using improved culture practices, routine sanitation and the use of chemotherapeutics, viral diseases have been far more problematic to manage [172], as specific vaccines are unable to be used as a method of prevention, due to the absence of a developed adaptive immune system in crustaceans [173].

Prevention and control of disease outbreaks mostly relies on strengthening the innate immune response to viral diseases. Therefore, understanding shrimp defence mechanisms in combination with different strategies can contribute to improve disease management. However, the molecular mechanisms involved in antiviral immune responses remain unknown for the majority of crustacean species [174]. Consequently, an understanding of viral entry and propagation in shrimp, as well as the interaction between host-pathogen at the cellular and molecular levels is crucial for the development of effective strategies to overcome shrimp viral infections [175].

Since RNAi's discovery, major proteins involved in the RNAi pathway, including Dicer and Argonaute, have been already identified in *P. monodon* [176-178], *L. vannamei* [179, 180] and *Marsupenaeus japonicus* [181] , confirming the existence of RNAi machinery in shrimp. In this scenario, genes from economically important shrimp pathogens, such as WSSV, YHV, TSV and Densovirus (DNV), have been targeted by sequence-specific dsRNA, and shown to be effective in promoting shrimp survival, as well as in interfering with viral replication (table 2.2). However, as several excellent reviews addressing this topic have been published in the past six years [168, 173, 181-184], only the most relevant facts will be recapitulated in the present review.

2.4.3.1. Stimulation of innate immunity and antiviral silencing by dsRNA

While sequence-specific effects of dsRNA that result in endogenous RNA degradation are widely conserved and probably present in most invertebrates [185], sequence-independent induction of antiviral immunity by dsRNA has long been thought to be exclusive to vertebrates [186]. This assumption was originally made based on the fact that genes homologous to IFNs or IFN receptors are absent in several fully sequenced invertebrate genomes, such as *Drosophila melanogaster* [187], *Caenorhabditis elegans* [188], *Anopheles gambiae* [189] and *Ciona intestinalis* [190]. Nevertheless, this concept has been reassessed since Robalino and collaborators demonstrated, *in vivo*, that long dsRNA can induce not only nonspecific innate antiviral immunity mediated by unrelated dsRNAs [191], but also potent sequence-specific antiviral response in *L. vannamei*, suggesting the possibility of dual stimulation of innate immunity and antiviral silencing by dsRNA in invertebrates [186]. According to the authors [191], the protection induced by unrelated dsRNA was overcome by increasing the viral load. This was the first evidence that lack of homologous genes does not rule out the existence of invertebrate immune systems analogous to those present in vertebrates. The principal finding was subsequently validated *in vitro* [192], as well as in other shrimp species: *P. monodon* [193-195] and *P. chinensis* [196].

Table 2.2 The applications of RNAi to investigate the interactions between shrimp and viruses.

Reference	Specie	Target gene	Delivery	Pathogen	RNAi inducer
[152]	*P. monodon*	YRP65	transfection	YHV	*in vitro* transcribed dsRNA
[133]		YHV-pro, hel, RdRP, gp116, gp65	transfection	YHV	*in vitro* transcribed dsRNA
[134]		YHV-pro	injection	YHV	bacterially expressed dsRNA
[154]		YHV-pro	injection	YHV	bacterially expressed dsRNA
[153]		YHV-pro, Rab7	injection	YHV	bacterially expressed dsRNA
[139]		vp15, vp28	injection	WSSV	siRNA
[142]		vp19, vp28	injection	WSSV	vector-based lhRNA
[144]		vp28	injection	WSSV	bacterially expressed dsRNA
[143]		Rab7, rr2	injection	WSSV	bacterially expressed dsRNA
[42]		vp28	oral	WSSV	bacterially expressed dsRNA
[159]		ns1, vp	injection	DNV	bacterially expressed dsRNA
[135]		ns1, vp	injection	DNV	bacterially expressed dsRNA
[148]		*Pm*Rab7	injection	LSNV	bacterially expressed dsRNA
[156]		GAV, β-actin	oral	GAV	bacterially expressed dsRNA
[212]	*L. vannamei*	RdRp	injection	YHV	vector-based lhRNA
[158]		YHV-pro	injection	YHV	bacterially expressed dsRNA

[141]		dnapol, rr2, tk-tmk, vp24, vp28	injection	WSSV	*in vitro* transcribed siRNA
[138]		duck (Ig)υH chain	injection	WSSV	*in vitro* transcribed dsRNA/chemic. synthesized siRNA
[155]		TBP, IE1	injection	WSSV	*in vitro* transcribed dsRNA
[146]		vp26, vp28	injection	WSSV	*in vitro* transcribed dsRNA
[132]		duck (Ig)υH chain	injection	WSSV-TSV	*in vitro* transcribed dsRNA
[127]		rr2, dnapol, ORF	injection	WSSV-TSV	*in vitro* transcribed dsRNA
[149]		Rab7	injection	TSV	bacterially expressed dsRNA
[151]		Lamr	injection	TSV	*in vitro* transcribed dsRNA
[136]		ORF1, 2, 3	injection	DNV	bacterially expressed dsRNA
[150]	*M. japonicus*	β-integrin	injection	WSSV	*in vitro* transcribed dsRNA
[145]		vp28	injection	WSSV	*in vitro* transcribed dsRNA
[140]		vp28	injection	WSSV	*in vitro* transcribed siRNA
[157]		vp28	injection	WSSV	bacterially expressed siRNA
[137]	*P. chinensis*	vp28, vp28-1, protein kinase	injection	WSSV	*in vitro* transcribed dsRNA

dnapol, DNA polymerase; DNV, *Densovirus*; hel, helicase; dsRNA, double stranded RNA; IE1, immediate-early protein; GAV, *Gill-associated Virus*; gp, glycoprotein; lhRNA, long hairpin RNA; LSNV, *Laem-singh Virus*; ns1, non- structural protein; ORF, open reading frame; pro, protease; Rab7, GPTase late endosomal protein; RdRp, RNA dependent RNA polymerase; rr2, ribonucleotide reductase; siRNA, small interfering RNA; TBP, TATA binding protein; tk-tmk, thymidine-thymidylate kinase; TSV, *Taura Syndrome Virus*; vp, structural gene; WSSV, *White Spot Syndrome Virus*; YHV, *Yellow Head Virus*; YRP65, *P. monodon* binding protein.

2.4.3.2. Different factors influencing RNAi silencing in shrimp

Apart from general activation of antiviral immunity and induction of specific RNAi silencing, Robalino et al. [186] also demonstrated that injection of siRNAs failed to induce a similar response. Despite being supported by Labreuche [197], such observation was further contested by a number of investigators, who successfully demonstrated that nonspecific [198] and specific [199, 200] protection can be mediated by the administration of siRNA. The inconsistency reported above can be explained by the employment of different methods to select and design the target fragments used in each assay.

The efficiency of antiviral response has also been proved to be dose [193, 198, 201] and target-dependent [186, 192, 194-196, 200, 202]. It has been repeatedly reported that RNAi targeting viral non-structural genes, such as ribonucleotide reductase small subunit (*rr*2), DNA polymerase (*dnapol*),thymidine kinase and thymidykate kinase (*tk-tmk*), protease (*pro*) and helicase (*hel*), are more likely to confer a higher degree of protection than those targeting structural genes (*vp*28 and *vp*26). This suggests that knocking down genes with lower degree of expression would be a more efficient strategy to inhibit viral replication by RNAi. However, despite this approach being frequently adopted [186, 192, 194, 195, 200] the above hypothesis should be interpreted with caution as several reports have shown that RNAi-inducing molecules designed against viral structural proteins can also provide strong viral inhibition [196, 203-205].

2.4.3.3. Improving protection by simultaneous knockdown of shrimp and virus specific genes

Apart from viral specific genes, targeting host endogenous genes involved in pathogen entry and replication, such as cell surface receptors and virus-binding proteins, have also been highly explored. Among the most frequently targeted are: Rab GTPase proteins [206-208] , β-integrin [209], Laminin receptor protein (*Lamr*) [210] and Lymphoid cell-expressed receptor [211]. However, considering the process of viral entry into a permissive cell is mediated by both cellular and viral factors, the co-administration of RNAi targeting viral and host specific genes, simultaneously, could potentially afford a better protection than when the same constructs are delivered individually. Such affirmation was recently validated by Posiri et al. [212], who showed that single injection of a combination of dsRNAs corresponding to the YHV protease and the endogenous *P. monodon* GTPase protein (*Pm*Rab7) resulted on significant lower cumulative mortality, than when the dsRNAs were delivered alone [193, 213].

Similarly, simultaneous silencing of *P. monodon* TATA box binding protein (*Pm*TBP), and the WSSV immediate-early protein (IE1) resulted in considerable lower viral load than when each gene was individually silenced [214]. Unexpectedly, similar effects were not observed by Attasart et al. [202], when targeting the *Pm*Rab7 and the early transcribed WSSV *rr*2 genes. Multiple injections of either combined dsRNAs or *rr*2-dsRNA alone similarly improved survival rates by approximately 95%, while only partial protection was afforded by nonspecific dsRNA (GFP) and *Pm*Rab7-dsRNA (approximately 55%). The discrepancy observed among studies can be easily explained by the fact that different target genes, viral load, shrimp size, dsRNA ratios and injection frequency were adopted by each author. Therefore,

despite the potential of simultaneous gene knockdown as an improved strategy in the control of virus infection, it appears that careful optimisation of several parameters is required in order to achieve enhanced results.

2.4.3.4. The ability of RNAi to induce preventive and therapeutic effect in shrimp

Several studies demonstrated that administration of pathogen-specific dsRNA/siRNA prior or simultaneously to viral challenge can considerably prevent replication of several virus species [192, 194, 199, 215, 216]. However, it has also been reported that delivery of RNAi-inducers to pre-infected animals can elicit a curative effect in shrimp [195, 212, 213, 217, 218]. According to the literature, such therapeutical responses can be greatly improved by simultaneous knockdown of viral non-structural and structural proteins [218], as well as viral and host genes [212].The ability of RNAi to confer curative effect in infected shrimp can be of great value in hatcheries, especially to prevent loss of precious brood stock from virus outbreaks. However, despite the promise of RNAi and its potential for protection and therapeutic treatment against shrimp viral disease, the development of practical and cost-effective strategies to deliver RNAi on a large scale is crucial for the successful application of RNA-technology in shrimp farms.

2.4.3.5. Seeking oral delivery of RNAi-based technology in shrimp

Oral administration by incorporating RNAi-inducing molecules in the feed is the most desirable delivery method in shrimp. In this context, Sarathi et al. [49] explored the possibility of inhibiting WSSV replication via ingestion by using

bacterially expressed dsRNA. *P. monodon* administered with pellet feed coated with inactivated bacteria containing over-expressed *vp*28-dsRNA or vp28dsRNA-chitosan complex nanoparticles presented a cumulative mortality of 32% and 63% when submitted to viral challenge. No survival was observed in shrimp that were treated with inactivated bacteria containing empty vector. This was the first report showing the feasibility of oral administration of dsRNA expressed in bacteria to protect shrimp against viral infection.

In contrast, Sellars et al. [215] found that administration of bacterially expressed dsRNA targeting the gill-associated virus (GAV) of *P. monodon* conferred protection when administered by muscular injection, but not when orally delivered as a feed component. Similarly, oral administration of dsRNA encoding the *P. monodon* β-actin showed no effect on shrimp survival, whereas 100% mortality was detected when delivered by injection, suggesting that injection of bacterially expressed dsRNA can successfully interfere with endogenous shrimp mRNA and exogenous viral RNAs. According to the authors, insufficient dsRNA delivery across the shrimp gut wall and dsRNA degradation by the gut digestive nucleases may be some of the factors that might have contributed to the failure of the oral delivery approach employed in the study. However, the authors did not measure the presence of dsRNA in the gut epithelium or any of the surrounding tissues. Perhaps it is pertinent in any feeding based studies that measurements of effective dsRNA levels be undertaken to confirm ‘delivery’ of the molecules.

2.4.3.6. The future of RNAi to control viral diseases in shrimp

Considering all the inconsistency reported in RNAi studies performed in shrimp, the development of a feasible and effective delivery method of RNAi

molecules into shrimp obviously remains a challenge. In the future, the employment of more standardized protocols would be highly desirable, as the lack of standardization in the assays makes meaningful comparison between studies almost impossible. Clearly, more research effort should be directed towards the development of oral RNAi delivery methods for use in shrimp farms. This matter will be discussed further in section 2.5.2.

2.4.4. RNAi technology applied to other crustaceans

Although RNAi technology has been widely used to assess gene function and therapeutic effects in fish and prawns, studies conducted in other economically important crustaceans, such as crabs and crayfish are still scarce.

The first study reporting the use of gene silencing mediated by RNAi in crayfish was performed by Liu et al. [219]. Silencing the anti-lipopolysaccharide factor gene (ALF) from freshwater crayfish *Pacifastacus leniusculus* significantly enhanced WSSV replication and viral *vp*28 transcript levels, both *in vitro* and *in vivo*, suggesting a protective role of ALF against WSSV in crustaceans.

Gene depletion mediated by RNAi was employed by Liu et al. [220] to explore the role of phenoloxidase enzyme (PO) in the immune defence of *P. leniusculus* against pathogenic *Aeromonas hydrophila*. Silencing of the prophenoloxidase acting system gene (proPO), the inactive precursor of PO, significantly reduced the levels of PO and phagocytic activity, as well as increased bacterial growth and the cumulative mortality of crayfish submitted to bacterial infection. In contrast, the opposite was observed following knockdown of the inhibitor of crayfish proPO activation cascade, the pacifastin gene. The results

altogether strongly indicated that production of PO is an important component in crayfish immune defence against this highly pathogenic bacterium.

The immune response to bacterial infection of red swamp crayfish *Procambarus clarkii* cell adhesion factor [221] and putative G protein-coupled receptor [222], called peroxinectin (*Pc*pxin) and HP1R gene respectively, were also recently investigated. Depletion of *Pc*pxin greatly reduced the survival rate of infected *P. clarkii*, demonstrating an involvement of this protein in crayfish immunity. Furthermore, it appears as though *Pc*pxin significantly regulates the expression of two antibacterial peptide genes (crustin1 and lysozyme) in response to *A. hydrophila* challenge, suggesting a possible role of *Pc*pxin in signalling pathway [221]. Silencing the HP1R gene, on the other hand, provided strong evidence for the participation of this molecule in crayfish susceptibility to *A. hydrophila* and *Vibrio alginolyticus*, as well as in bacterial clearance, total haemocyte count and PO activity [222].

2.4.5. The early stages of RNAi application in molluscan aquaculture

Unlike fish and crustaceans, the application of RNAi technology in shellfish aquaculture remains in its infancy. Up to now, the majority of the work involving the use of RNAi in marine molluscs has focused on providing information on functional analysis of genes involved in gonad maturation [223, 224] and shell formation [225-227]. However, despite the fact that substantial economic and environmental impacts are caused worldwide by the emergence and spread of new infectious diseases in the shellfish aquaculture industry [228], the potential use of RNAi to develop treatment strategies against such diseases has been barely explored.

The only study covering aspects of molluscan immune response against pathogens was recently performed by Wang et al. [229], where RNAi was employed to validate the existence of Zhikong scallop (*Chlamys farreri*) TLR (*Cf*TLR) signalling pathway, as well as its involvement in bivalve immune defence. dsRNA-suppression of *Cf*TLR not only downregulated four other key genes involved in TLR signalling pathway, but also the immune-related effector gene G-type lysozyme. Conversely, the expression levels of two major antioxidant enzymes (SOD and catalase) were significantly up-regulated. Moreover, lower apoptosis level and higher cumulative mortality was detected under bacterial challenge with *Listonella anguillara*. The results collectively indicate that several immune responses, such as apoptosis, antibacterial and antioxidant activity, can be activated by the TLR signalling pathway in scallops.

Until recently, a possible reason hampering the expansion of RNAi studies in shellfish was the lack of a bivalve primary cell line, which only enabled the development of *in vivo* experiments. However, an RNAi assay recently conducted on primary cells of clam *Meretrix meretrix* digestive glands showed, for the first time, that this technology can be successfully employed on marine bivalve cells [230], opening new doors for further research on the mechanisms underlying disease transmission and host-pathogen interaction. For example, RNAi could be explored to enhance research efforts directed to better understand the biology and replication of new and emerging viruses, such as the new strain of *Crassostrea gigas Ostreid herpesvirus* (OsHV-1µVar), which was first detected in France 2008 and subsequently spread across Europe, causing massive mortality outbreaks in several producing areas [231, 232].

Apart from gene function discovery, RNAi could also have the potential to be employed to activate the RNAi-mediated antiviral defence in molluscs, since such

organisms lack an adaptive immune response to pathogens [233] and the development of traditional vaccination strategies are not possible for them. However, the fact that mollusc culture systems are mainly performed in open environment, finding a feasible and effective delivery strategy would be the main obstacle to be overcome before RNAi therapy finds practical application in shellfish aquaculture.

Besides the characteristics of the culture systems, bivalve's physiology, such as filter feeding habit and body enclosed by a pair of hard shells, exclude the possibility of delivering RNAi constructs through feeding or injection. As a result, immersion would be the only realistic delivery strategy, which would involve having to transfer the animals to some source of temporary soaking reservoir. However, as the gene silencing effect by RNAi is temporal and its duration is determined by various factors [234], the frequency of bathing required to maintain the protective effect could make such practice economically unviable. A more practical use of RNAi could be found in gastropods farming systems, such as abalone, which are reared in land-based tanks and are usually fed artificial feeds, enabling the incorporation of RNAi-inducing molecules in their diet. In summary, considering the massive impact that infectious diseases have in shellfish aquaculture worldwide, we believe that more research effort has to be devoted to explore the possibilities of employing RNAi technology as prophylactic strategy in shellfish farms.

2.5. RNAi limitations and further perspectives

2.5.1. General limitations

RNAi has emerged as a powerful tool to manipulate gene function and is considered a promising strategy to control disease pathogens in aquaculture.

However, despite the excitement about this remarkable biological process for sequence specific gene regulation, there are a number of hurdles and concerns that must be overcome before RNAi therapy finds practical application in aquaculture. These include the potential for off-target effects, triggering innate immune responses and, most importantly, effective delivery *in vivo*.

Specific off-target activity occurs when partial sequence homology allows siRNA/shRNA to degrade mRNA for genes that are not the intended silencing targets, which can complicate the interpretation of phenotypic effects and potentially lead to unwanted toxicities [235]. Designing optimal siRNAs duplexes or shRNA is one of the main strategies to minimize the occurrence of off-target effects. According to the literature, many parameters can influence the specificity of siRNAs/shRNA, such as the elected target region [236], the size [108], the starting nucleotide [237], the GC content [237], the thermodynamic properties [238] and the presence of internal repeats or palindromes [239], among others. Thus, a number of computational design tools have been developed to accurately and systematically evaluate RNAi off-target effects between siRNA sequences and target genes on a transcriptome-wide scale [240-242].

Apart from effective design of RNAi molecules, the application of appropriate controls, such as endogenous positive controls and scrambled, mismatched and non-targeting negative controls, is also strongly recommended to ensure validity of RNAi data [243]. The use of multiple individual siRNAs targeting the same gene can also be implemented to confirm the specificity of RNAi experiments, as different siRNAs to a single target gene, with comparable gene silencing efficacy, should induce similar changes in gene expression profiles or phenotypes [244].

Nonspecific off-target effects is another factor compromising the specificity of RNAi experiments, where certain sequence motifs in long dsRNAs, siRNAs and shRNAs can trigger type I IFN response in higher vertebrates [107, 109, 245, 246] and fish [50, 146, 153]. Considering IFN is a critical nonspecific viral defence mechanism，a cellular IFN response should be given special attention in studies of RNAi targeting viruses, where reduced replication in transfected cells is often taken as indicative of successful specific interference [146]. Therefore, it is highly recommended to check for this effect by analysing the level of expression of IFN pathway-related genes by northern blot or quantitative reverse transcription PCR (qRT-PCR) analysis [247]. The phenomenon of innate antiviral immune reactions activated by dsRNA was first thought to be restricted to vertebrates, as genes encoding homologues of interferons and IFN-regulated genes were absent in sequenced invertebrate genomes [197]. However, many studies have shown that dsRNA can effectively block viral disease progression in shrimp, suggesting that dsRNA can activate antiviral immunity in crustaceans through two pathways that utilize sequence-independent [186, 191-193, 196] and sequence-dependent dsRNAs [199, 213, 218]. Therefore, careful design of RNAi constructs, as well as the use of appropriate controls should be employed when assessing RNAi-mediated silencing in crustaceans.

As siRNA/shRNA relies on the endogenous miRNA machinery to achieve potent target silencing, excessive levels of exogenous RNAs can saturate one or more components of the endogenous RNAi pathways, causing potential toxicity and fatalities in animals [248]. Therefore, monitoring both mRNA and protein levels can be beneficial, as high doses of ectopic siRNA/shRNA may function as miRNAs and suppress translation without significantly affecting mRNA levels [249]. Moreover, titration of the siRNA to the lowest possible level is highly encouraged as RNAi is

often extremely effective at minimal concentrations [112]. The use of relatively low doses of siRNA/shRNA is also a commonly employed strategy to increase silencing specificity by significantly reducing the occurrence of specific and nonspecific off-target effects [109-112, 250].

Besides off-target effects, the delivery of RNAi silencing molecules to specific tissue or cells remains one of the most important challenges to the development of safe and effective *in vivo* RNAi therapy, as siRNAs/shRNAs are known to present rapid excretion, low stability, nonspecific tissue distribution, poor cellular uptake and inefficient intracellular release [158]. Chemical modifications, including changes in the backbone, replacement of individual nucleotides with nucleotide analogues and addition of conjugates, can be introduced into the RNAs oligos to increase their stability in the extracellular and intracellular environments [251]. However, due to their negative charge and size, naked siRNAs are usually unable to penetrate cellular membrane and reach the cytoplasm of the target cell, which is crucial for effective silencing [252]. Therefore, appropriate delivery systems are required to increase cellular accumulation of siRNAs and facilitate the release from endosomes to the cytosol [253]. Many delivery systems, such as nanoparticles, cationic lipids, calcium phosphate, antibodies, cholesterol, aptamers and viral vectors [254], have been widely tested as the accessibility of different tissue types, various delivery routes and a variety of pharmacological requirements makes it impossible to have a general *in vivo* delivery system suitable to every scenario of RNAi delivery. Consequently, inappropriate selection of a delivery vector can reduce gene-silencing activity, as well as enhance undesirable off-target effects and cause toxicity [255].

With regards to poor dsRNA uptake, a newly identified transport protein, named SID-1, can hold the key for enhancing dsRNA internalization into cells. SID-1 is a widely conserved dsRNA channel that selectively and specifically transports

dsRNA into cells and is essential for systemic RNAi [256]. In this context, cells over-expressing SID-1 could remarkably facilitate systemic spread of exogenous dsRNA and thereby elicit a superior RNAi response. Therefore, considering that SID-1 homologues have been recently reported in fish [144] and shrimp [197] species , over-expression of SID-1 associated with the administration of exogenous viral- dsRNA could significantly improve the RNAi-antiviral mechanism in those organisms.

Low transfection efficiency can also be a major problem when gene inhibition by the use of siRNAs is attempted in fish cells [257], as low transfection rates result in only a fraction of the cells performing RNAi [138]. According to Xie et al. [41], cationic liposomal reagents, such as Lipofectine and Lipofectamine™ 2000, exhibited higher transfection efficiency than calcium phosphate. Consequently, lipid carriers have been the most commonly employed delivery system in fish cell lines transfection assays [41, 42, 46, 48, 52, 144, 147, 150, 155]. However, it should be taken into account that their application must be optimized for each type of target cell, as cationic liposomes have been reported to cause toxicity and induce nonspecific IFN response [258, 259].

Another important practical issue that needs to be taken in consideration is the ability of some viruses to escape RNAi-mediated suppression, especially through mutation of the target region and by encoding viral suppressors [260]. Simultaneous targeting of multiple viral sequences with a pool of siRNAs is one of the strategies to prevent the emergence of resistant virus [261]. Administration of long hairpin RNAs (lhRNA) is another commonly employed alternative as generates multiple siRNAs from a single precursor without inducing IFN response [262]. However, it needs to be taken into consideration that over-expression of multiple siRNAs may result on undesirable saturation of endogenous RNAi pathway [173].

2.5.2. RNAi limitations directly applied to aquaculture

Apart from all the issues cited in the section above, which are general RNAi limitations regardless of the target system, the selection of an appropriate delivery method suitable for each aquaculture rearing system is one of the main obstacles to make RNAi a realistic tool in industrial scale.

Open sea-cage aquaculture, for example, would face similar limitations as open-water shellfish farms (already discussed in section 2.4.5). Due to environmental reasons, incorporation of RNAi-inducing molecules on the feed is not a viable practice, as fish are kept in floating sea cages or net-pens, which allow the uneaten feed to accumulate on the bottom of the ocean, becoming available to non-target organisms. Submitting the fish to immersion baths in temporary enclosed structures could be a strategy. However, this practice has already been shown not cost effective in open water fish farms. A good example is the case of amoebic gill disease (AGD) in Atlantic salmon farms in Tasmania, Australia [3]. The only effective treatment available for the disease is bathing the fish in fresh water, which involves transferring all the animals from a net-pen into a freshwater containing canvas liner and, after a period of three hours, releasing them back into the underlying net-pen [23]. Despite effective, this procedure is not considered a viable long-term management solution, as it accounts for up to 20% of production costs [13]. If a similar practice was to be introduced as RNAi delivery strategy, additional operating costs would be required to manage the soaking solution, as it cannot be simply discharged in ocean. Therefore, we believe that RNAi mediated by immersion should not be considered a realistic practice in open sea-cage aquaculture. Despite laborious and time consuming, injection could a viable strategy, as vaccines and antibiotics are already often delivered into the fish as a common aquaculture practice. In this case,

the RNAi silencing molecules could be incorporated in the already administered additives, without adding further operational costs. However, the possibility of the substances interfering with the stability and effectiveness of the RNAi precursors may hold back the use of such administration method. Thus, considering all the implications associated with RNAi delivery in open-water fish farms, as well as the fact that fish can mount adaptive immune response enabling the use of classic vaccination strategies, we believe that RNAi technology would find a better use as a complementary tool to assist in the identification and validation of new treatment targets, rather than a therapeutic strategy in open sea-cage aquaculture.

The administration of RNAi therapeutics to land-based systems has certain advantages over open-environment aquaculture systems, as it permits easier waste management and direct access to animals and laboratory facilities. In shrimp farms, the high number of animals reared in pond/tanks makes injection a non-realistic strategy for commercial field applications. Soaking the organism in solution containing *in vitro* synthesised RNAi precursors is an effective and non-invasive strategy widely used in RNAi studies on shrimp. However, such strategy can also become increasingly expensive and laborious as large amounts are usually required to ensure continuous suppression of the target gene. Additionally, synthetic RNA duplexes are known to limit the RNAi-effective time in shrimp due to their very short half-life when administered *in vivo* [263]. Therefore, further studies are necessary to construct high expression vectors and better transfection approaches of RNAi constructs in shrimp *in vivo*. A simple and cost effective system to generate large amounts of dsRNA *in vivo* was proposed by Ongvarrasopone et al. [264] using an RNase III-deficient *E. coli* strain (HT115). Through this method, bacteria expressing dsRNA can be either submitted to a purification step to isolate the required dsRNA or be directly incorporated into pellet feeds to be orally delivered.

The protocol can also be modified to successfully produce large quantities of stable vector-based hairpin dsRNAs [265]. The delivery of such constructs via ingestion is currently considered the most feasible delivery method for inland aquaculture operations [168]. Nevertheless, the only two studies that have demonstrated the application of this methodology on shrimp showed very conflicting results [49, 215], indicating that the development of a viable and effective delivery method of RNAi molecules into shrimp is still on early stages. The lack of a permanent shrimp cell line is another factor hampering a better understanding of the RNAi mechanism in shrimp, as well as the host-pathogen interaction at the molecular level, which is very critical for the development of strategies against pathogens [168].

A potential approach that could be employed in the future to overcome some of the delivery issues associated with the application of RNAi in industry-scale would the generation of transgenic organisms that express RNAi molecules targeting key conserved region of specific pathogens [125]. However, the implementation of genetic engineering technology to food animals will certainly raise further environmental and safety-related issues.

2.6. Conclusion

The studies presented in the present review demonstrate that the success of RNAi experiments in aquatic farmed animals can be highly influenced by several parameters, such as selected target gene [40, 186, 192, 196, 202], nucleotide sequence [45, 199], RNAi molecule [41, 186], length of the fragment [192, 197], target tissue [266, 267], dosage [223, 226, 268, 269], delivery route [215], transfection reagents [41], administration frequency [202] and target pathogen [270, 271]. These results indicate that, despite RNAi technology being considered a

promising tool to control disease pathogens of economically important species, many hurdles still have to be overcome in order to develop a safe and effective method for delivering RNAi *in vivo*. Higher stability of RNAi molecules, as well as specific and controllable long-term silencing effects, are some of the issues that need to be addressed before RNAi therapy finds practical application in aquaculture. Also the implementation of efficient and cost effective delivery methods, without negatively impacting the environment remains a crucial challenge for the establishment of RNAi-based technology at an industrial scale. Further progress in understanding the RNAi machinery at the molecular level, as well as the interaction between host-pathogen and the mechanisms by which these pathogens propagate are also highly desirable.

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CHAPTER 3: Experiment - 1

Towards the application of RNAi against Atlantic salmon Amoebic gill disease: Identifying key components of the RNAi machinery in closely related species of the genus *Neoparamoeba*

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Abstract

We utilised a *Neoparamoeba pemaquidensis* transcriptome database to identify putative proteins involved in the RNAi-pathway. A distinct Dicer homologue was not confirmed for *N. pemaquidensis*, however a single contig containing a putative DEXDc helicase domain (*Np*DEXDc) shared significant similarity against Dicer proteins from several species. A protein homologue with the expected Piwi and PAZ domains for eukaryotic Argonaute (Ago) was also identified in the database (*Np*AGO-2). Pairwise alignment of the open reading frame showed high level of homology against proteins from the Ago subfamily. Dicer and Ago candidates were significantly up-regulated when dsRNA and esiRNA, respectively, were administered into the amoeba culture, evidencing their involvement in the canonical gene-silencing pathway. The presence of an active Dicer in *N. pemaquidensis* was also corroborated by an RNAse III assay, which showed complete degradation of

dsRNA followed incubation in *N. pemaquidensis* lysate. Functional evidence for the presence of an active RNAi machinery was also supported by esiRNA-mediated gene silencing. Surprisingly, while transfected esiRNAs targeting *N. pemaquidensis* β-actin and EF1-α resulted in successful downregulation of either target gene, *in vitro* transcribed dsRNA failed to significantly knockdown the same targets when delivered by soaking (20 µg/mL). However, as a slight reduction in the expression level of both genes was detected at 24 h, we believe the silencing efficiency could be improved by using higher doses or transfection techniques. Protein candidates encoding putative conserved motifs found in Drosha and Piwi subfamilies were also identified, suggesting that miRNA and piRNA pathways could be present in *N. pemaquidensis*. This study provides insights into understanding the mechanism of RNAi in *N. pemaquidensis*. Such findings represent a major step towards transferring this technology to the closely related species *Neoparamoeba perurans*, the causative agent of amoebic gill disease in farmed Atlantic salmon.

Keywords: amoeba; aquaculture; RNA interference; Dicer; Argonaute

3.1. Introduction

RNA interference (RNAi) is a natural regulatory mechanism of most eukaryotic cells that uses small double-stranded RNA (dsRNA) molecules as triggers to direct homology-dependent control of gene activity [54]. Dicer and Argonaute (Ago) are the two main core proteins involved in the RNAi pathway. Dicer is an RNAse III endoribonuclease enzyme that has the ability to recognize and cleave endogenous or exogenous dsRNAs into 21-25 nt small-interfering RNAs (siRNAs) [272]. Ago, on the other hand, is the main component of the RNA-induced silencing complex (RISC) [273]. RISC incorporates and unwinds the duplex siRNA, using the antisense strand as a template to select complementary mRNA substrates for degradation [274].

The presence of highly conserved regions within both protein families has greatly facilitated the identification of RNAi-related orthologs through comparative genomic analysis, particularly in species without an annotated genome. Dicer are typically large multidomain proteins containing DEXDc DEAD-like helicase, DUF283, PAZ, two RNase III (a and b) and an additional dsRNA binding domain (dsRBD) [275] (Figure 3.1A). Ago-family proteins, on the contrary, have two main signature domains (PAZ and Piwi), as well as a N-terminal and a MID region flanked by PAZ and Piwi [276] (Figure 3.1B).

The Ago protein family is subdivided into two subfamilies: Ago and Piwi clade [277]. While siRNAs and microRNAs (miRNAs) associate with Argonaute proteins of the Ago clade, recent studies have identified a third class of small RNAs that binds to members of the Piwi subfamily: Piwi-interacting RNAs (piRNAs) [278].

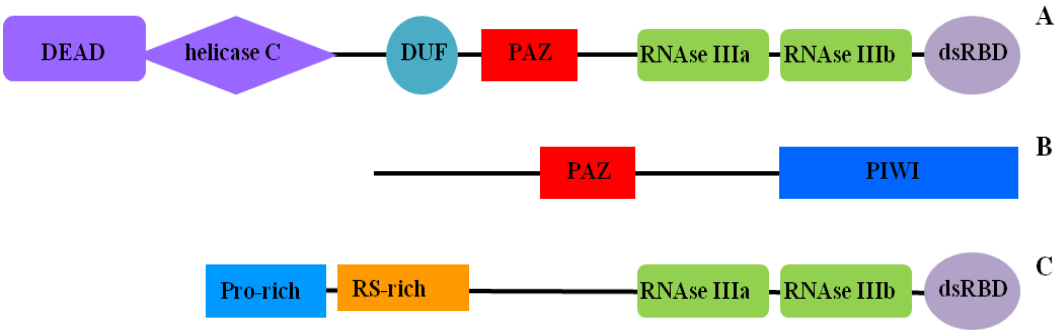

Figure 3.1 Schematic representation of the domain architectures found in eukaryotic RNAi-related proteins. (A) Dicer, (B) Argonaute and (C) Drosha. Individual protein domains are indicated by colours.

Amoebic gill disease (AGD), caused by the protozoan amoebae *Neoparamoeba perurans*, is emerging as one of the major challenges hampering the sustainability of Atlantic salmon (*Salmo salar*) aquaculture industry worldwide [8]. In Tasmania (Australia), where it was first reported nearly three decades ago [279], AGD remains the most significant health concern affecting the local Atlantic salmon production [36]. Despite years of research, control of AGD is still restricted to bathing infected fish in freshwater [280], which costs the industry millions of dollars per year in terms of loss of production and treatment costs [281]. Therefore, there is a pressing need to develop alternative treatment strategies that reduce the need of freshwater bathing. One such technology that has the potential to fulfil this need is RNAi. Due to the sequence-specific ability to silence target genes, the analysis of gene function through RNAi could be valuable for screening genes involved in amoeba virulence and mechanisms of pathogenecity. This could greatly assist in the identification and characterization of new targets for the development of treatments against AGD.

However, as the RNAi pathway is known to be repeatedly lost during the evolution of certain protozoan parasites species [54, 282, 283], the presence of an active RNAi pathway in *N. perurans* needs be confirmed before such technology can

be used in efforts to control AGD. Nevertheless, as the *in vitro* culture of *N. perurans* has only recently been achieved [36], the aim of the present study was to provide functional and comparative genomic evidence for the presence of functional RNAi machinery in the closely related *Neoparamoeba pemaquidensis*, a non-infective strain of the genus *Neoparamoeba.*

3.2. Material and Methods

3.2.1. Dicer and Argonaute candidates

N. pemaquidensis Dicer and Ago sequences were obtained from an Illumina Next Generation sequencing effort (M. Cook, Unpublished data). Annotated nucleic acid sequences were checked for open reading frames (ORF) using the online software ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) and the selected reading frames translated into fasta protein sequences. The Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify Dicer and Ago conserved domains by comparing translated protein sequences against the UniProtKB/Swiss-Prot database (www.us.expasy.org/toolsblast) and the National Center for Biotechnology Information (NCBI) non redundant (nr) protein database (www.ncbi.nih.nlm.gov). An e-value cut-off of $1.0e^{-5}$ was applied to identify strongly supported domains. The highest scoring Dicer and Ago matches were aligned using ClustalW2 to compare sequences features and similarities (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

3.2.2. Amoeba culture conditions

N. pemaquidensis, the non-infective strain of the causative agent of AGD, was used for analysis in the current study. Trophozoites originally obtained from AGD-infected salmon gills were grown on malt-yeast-seawater agar plates and kept at 16 °C. On the day before each experiment, amoebae were detached from the monolayer agar culture using a transfer pipette and sterile seawater. The amoebae suspension was then submitted to three-low speed centrifugation steps (400 x g, 3 min) to remove bacterial load naturally present in the agar culture. The final amoebae pellet was resuspended in sterile seawater and evenly transferred to 24-well culture plates (Nunclon^{TM} Delta Surface) to a final concentration of 10^5 amoeba/mL/well. The number of cells was estimated using a haemocytometer. Plates were incubated at 16 °C throughout the experimental period.

3.2.3. RNA isolation and reverse transcription

Prior to RNA isolation from *N. pemaquidensis*, seawater was removed from target wells and the amoebae grown in monolayer rinsed twice with sterile seawater to eliminate floating debris and excess bacteria. Trophozoites were then detached from the wells by adding 1 mL of TRIZOL® (Invitrogen) and the homogenized sample subsequently transferred onto a QIAshredder spin column (QIAGEN) to ensure complete disruption of cells. RNA extraction was then performed using the RiboPure^{TM} kit (Ambion), as per the manufacturer's protocol. RNA was quantified using a Nanodrop ND-1000 Spectrophotometer and its integrity verified on a 1.5% TAE agarose gel. Contaminating DNA was digested with TURBO DNA-free^{TM}

(Ambion) and total RNA reverse transcribed to cDNA using Superscript™ III Reverse Transcriptase and oligo(Dt) primer (Invitrogen).

Table 3.1 Primer sequences used for dsRNA *in vitro* transcription (ds) and qRT-PCR (q).

primer	nucleotide sequences (5'-3')
ds_β-actin.5'	GATACTAGTACCTTCAACACCCCCGCCATG
ds_β-actin.3'	AACGCTAGCTAGGACTTCTCGAGGGCAGAG
ds_EF1α.5'	GATACTAGTGCCGGAAAGTCCACCACCACT
ds_EF1α.3'	AACGCTAGCTGTACCCGATCTTCTTCAAGA
ds_ludiferase.5'	GATACTAGTATGGAAGACGCCAAAAACATA
ds_luciferase.3'	AACGCTAGCAACCCCTTTTTGGAAACAAAC
q_β-actin.5'	CAATCCAAGCGTGGTATCCT
q_β-actin.3'	GCTCGTTGTAGAAGGTGTGG
q_EF1α.5'	GTACAAGGGTCCCACTCTCCC
q_EF1α.3'	AGCGAAGGTGACAACCATAC
q_*Np*Drosha.5'	CATCCCAGTTTGACATCTGCT
q_*Np*Drosha.3'	TCTGTGTTCCCAATTTGAAGG
q_*Np*DEXDc.5'	ACCTCTTGTTTCCCAGCAGA
q_*Np*DEXDc.3'	AACAGAACGAGAAGGGCAGA
q_*Np*AGO-2.5'	TTCTGGACGACGAGGAAGAC
q_*Np*AGO-2.3'	CATCATCAAGGAGAGCGACA
q_*Np*Piwil-1.5'	TCGCTTGTGGGTCTGTTATCT
q_*Np*Piwil-1.3'	AAGTGGCTTTGTCGTGCTG
q_*Np*Piwil-2.5'	TCAAACCAAAGGGAGAAACG
q_*Np*Piwil-2.3'	GGCTCCAAAAACCTCACAAA
q_*Np*PiwiS.5'	CAAAGATGGGTTGAAGGTG
q_*Np*PiwiS.3'	GCAGTCATGTGATTGGAGG

3.2.4. Preparation of DNA template for dsRNA synthesis

Primers specific for *N. pemaquidensis* β-actin and EF1-α, as well as the control gene luciferase, were designed using Primer 3 software (http://www-genome.wi.mit.edu/cgi-bin/primer3_www.cgi) and the presence of secondary structures, such as hairpin and dimmers, assessed using Net Primer online software (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.htlm) (Table 3.1). Amoeba sequences were obtained through sequence analysis of a normalized

EST library (data not published). Target fragments were amplified using Phusion DNA Polymerase (Invitrogen) and cloned into pAquire vector (Gene Works), prior to subcloning into L4440 vector, which is bidirectionally flanked by T7 promoters. The resulting L4440 plasmid constructs were then verified by restriction analysis and DNA sequencing.

3.2.5. *In vitro* transcribed dsRNA

In vitro transcribed dsRNAs were generated using MEGAscript® T7 kit (Invitrogen), according to the manufacturer's instructions. Briefly, the DNA fragments previously cloned into the double T7 L4440 vector were linearized by restriction digestion with NheI and SpeI (New England BioLabs), in separate reactions, and complete linearization verified by agarose gel electrophoresis. Linear plasmids were then purified using the QIAquick® PCR purification kit (QIAGEN) and used as a template to synthesize the corresponding dsRNAs. Following transcription and annealing, the reaction was incubated for 1h at 37 °C with Turbo DNA-free™ (Ambion) and RNase A (QIAGEN) to digest template DNA and remaining single stranded RNA, respectively. The dsRNAs were purified using the RNA clean-up protocol from the RNeasy® Kit (QIAGEN) and visualised on a 1% agarose gel to confirm size product and integrity. Concentration of each dsRNA was determined using a Nanodrop ND-1000 Spectrophotometer.

3.2.6. Endoribonuclease-prepared siRNA pool

The ShortCut® RNAseIII kit (New England Biolabs) was used to convert *in vitro* transcribed dsRNAs into a heterogeneous mix of siRNAs (18-25 bp).

Approximately 10 µg of each dsRNA preparation was incubated with *Escherichia coli* RNAseIII at 37 °C for 20 min, according to the manufacturer's protocol. Following digestion, enzyme activity was stopped by addition of 10X EDTA and the resulting endoribonuclease-prepared siRNA pool (esiRNA) purified using the PureLink™ miRNA Isolation kit (Invitrogen). Successful dsRNA cleavage was confirmed by gel electrophoresis and esiRNA quantification determined by Nanodrop ND-1000 Spectrophotometer.

3.2.7. Validation of up-take of the RNAi-trigger

Fluorescein-siRNA transfection control (New England Biolabs) was used to confirm if RNAi duplexes were able to reach the amoeba cytoplasm either when delivered by transfection or directly administered into the culture. For this purpose, amoeba were seeded on 24-well culture plate at a density of 10^5 cells/well and kept at 16 °C, as described in section 3.2.1. Transfection of the fluorescent control was performed with Lipofectamine® RNAiMAX reagent (Invitrogen). Opti-MEM® I Reduced Serum Medium (Invitrogen) was used to dilute the transfection reagent and siRNA. Both mixtures were then combined and incubated for 5 min at room temperature, before addition to each well at a final concentration of 10nM. The same procedure was repeated without the addition of Lipofectamine® RNAiMAX reagent to ascertain if the fluorescent control would be incorporated into the amoeba cytoplasm through phagocytosis or passive up-take. Detection of fluorescent control was verified at 24h post transfection using a Zeiss Axio Observer inverted microscope under Fluorescein Isothiocyanate (FITC) filter set (excitation 495 nm, emission 516 nm). Images were acquired with a Zeiss Axiocam CCD camera and Zeiss AxioVision 4.8.1 software.

3.2.8. Gene silencing experiments

At 24 h prior to each experiment, the amoebae were transferred from agar plates to liquid culture and evenly split into 24-well culture plates (Nunclon™ Delta Surface) at a density of 10^5 amoeba per well, containing 1 mL of filtered seawater. Each treatment was performed in quadruplicate with each replicate comprising the trophozoites from three wells (3x 10^5) to ensure high RNA yield downstream processing. *In vitro* transcribed dsRNA encoding *N. pemaquidensis* β-actin and EF1-α, as well as the nonspecific control luciferase, were directly incorporated into the amoeba culture to obtain a final concentration of 20 μg/mL. The selected dsRNA dose was based on previous experiments performed in our laboratory using bacterially expressed dsRNA (Chapter 4). A second control group containing only amoeba was incorporated into the experimental design. A similar experiment in which the trophozoites were treated with esiRNAs corresponding to the same target sequences and control was also performed. Using methodology previously described in section 3.2.7, each pool of esiRNAs was either transfected or directly added into the culture at a concentration of 10 nM. Amoebae exposed to RNAi duplexes were cultured under standard *in vitro* conditions and sampled at 0, 24, 48 and 72 h post dsRNA/esiRNA administration.

3.2.9. Total RNA extraction and semi quantitative RT-PCR

At each sampling point, the amoebae were collected from target wells as detailed in section 3.2.2. dsRNA/esiRNA-treated amoebae were resuspended in TRIZOL® (Invitrogen) and total RNA isolated using RiboPure™ kit (Ambion), followed by Turbo DNAse-treatment (Ambion) and reverse transcription

(Invitrogen). As gene silencing efficiency may vary among replicates, pooled cDNA samples were obtained by combining cDNA aliquots from each replicate of a given treatment group. Each cDNA mix was used as PCR template in a reaction containing GoTaq® (Promega) and target genes' specific primers. The temperature profile for PCR amplification was performed as followed: 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min; and a final extension at 72 °C for 7 min. PCR products were visualized on agarose gel and mRNA expression levels of target genes evaluated by comparing band intensity between treated and control (luciferase) samples, at each time point.

3.2.10. Quantitative real time PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed on a ViiA7 Real-Time PCR System (Applied Biosystems), to determine whether administration of RNAi duplexes targeting the *N. pemaquidensis* β-actin and EF1-α would significantly downregulate target gene mRNA expression levels. qRT-PCR primers were designed to amplify regions of target mRNA external to the segment targeted by each dsRNA (Table 3.1). Primer sets were optimised and analysed by agarose gel electrophoresis to confirm a single specific PCR product. Dissociation curve analysis was also performed to assess non-specific primer–dimer amplification. Reactions were carried out in triplicate, each containing 3 µL of diluted cDNA, 2x SYBR® Green PCR Master Mix (Applied Biosystems), 0.5 mM forward and reverse primers and water to 10 µL. The amplification profile consisted of an initial hold stage (50°C for 2 min, 95°C for 10 min), followed by 40 cycles of 95°C for 15 s and 60°C for 1 min and a final dissociation according to the manufacturer's instruction (95°C for 15 s, 60°C for 1 min and 95°C for 15 s). The comparative cycle threshold (Ct) method

was used for relative quantification of gene expression [284]. This method involves comparing the Ct values of treated samples with a calibrator, which are normalized to an endogenous housekeeping gene. Calculations were performed using the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = [(Ct_{\text{target (unknown sample)}} - Ct_{\text{end.control (unknown sample)}})] - [(Ct_{\text{target (calibrator sample)}} - Ct_{\text{end. control (calibrator sample)}})]$. Percent knockdown was estimated through $100x\ (1 - 2^{-\Delta\Delta Ct})$. When β-actin knockdown was assessed EF1α was regarded as the endogenous control and *vice versa*. The control group containing only amoeba was considered as calibrator. To check if amplification efficiency of target genes were approximately equal to the endogenous control, five point standard curves were created and primer set efficiency calculated from the formula $E = 10^{-1/slope}$. Differences in β-actin or EF1-α expression levels within treatments, at each sampling period, was calculated by one-way analysis of variance (ANOVA) using the R version 2.14 software (R Development Core Team 2007). Values were considered to be significant at $p < 0.05$. All numerical data were expressed as the mean ± standard error.

3.2.11. Up-regulation of Dicer and Argonaute in response to RNAi duplexes

Up-regulation of *N. pemaquidensis* Dicer and Ago candidates following the administration of dsRNA and esiRNAs, respectively, was investigated. For this purpose, the same experimental parameters used in the silencing assays (section 3.2.6) were employed. However, only RNAi duplexes targeting EF1-α were administered in this instance. Samples were collected at 0 and 24 h and relative quantification of target mRNA transcripts calculated by the $2^{-\Delta\Delta Ct}$ method, using β-actin as endogenous control (as detailed in section 3.2.8). The qRT-PCR primers used to amplify *N. pemaquidensis* Dicer and Ago are listed in Table 3.1.

3.2.12. RNAse III activity assay

The ability of *N. pemaquidensis*' Dicer to recognise and cleave dsRNA was assessed by RNAse III activity assay, as described by Abed and Ankri [285]. Briefly, approximately 2 µg of *in vitro* transcribed dsRNA was incubated for 1 h, at 37 °C, with 5 µg of *N. pemaquidensis* protein lysate in a 20 µL reaction containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 25 µg/mL bovine serum albumin. The lysate was prepared with Nonidet P-40 1% in phosphate-buffered saline (PBS). Efficient degradation of dsRNA by Dicer present in the amoeba lysate was verified on a 2% agarose gel. Incubation with serially diluted lysate was also performed to confirm reduction of degradation effectiveness. The addition of 10x EDTA prior dsRNA incubation was carried out to validate Dicer inactivation.

3.3. Results

3.3.1. Dicer and Argonaute homologues

BLAST analysis of the *N. pemaquidensis* transcriptome database revealed two partial amino acid (aa) sequences containing conserved domains found in Dicer (see Appendix for sequences). Multiple sequence analysis of each candidate with the corresponding best-scoring hits from the BLAST output can be observed in Figure 3.2. The first candidate, a 127 aa long fragment containing a RNAse III C- terminal domain (RIBOc) (Figure 3.2A), shared high level of homology with *Homo sapiens* (Q9NRR4; 34% identity, 5^{e-11}) and *Macaca mulatta* (AAZ80928.1; 36% identity, 7^{e-11}) Drosha (Figure 2B), a Class 2 RNAse III enzyme involved in the miRNA

pathway. The second candidate (220 aa), on the other hand, was highly related to the DEXDc helicase domain (Figure 3.2C) of *Oryza sativa* Dicer 3b (Q7XD96; 30% identity, 8^{e-08}) and 4 (A7LFZ6; 27% identity, 1^{e-06}) homologues (Figure 3.2D). Dicer candidates 1 and 2, from this point forward, will be called *Np*Drosha and *Np*DEXDc, respectively.

Similarity search of annotated translated protein sequences also revealed four ORFs covering conserved regions of the Ago superfamily (see Appendix for sequences). Ago candidates 1, 2, 3 and 4, each contain 1046, 668, 70 and 60 aa, respectively. Candidates 1 and 2 comprise both Piwi and PAZ domains (Figure 3.3A and 3.4A), while candidate 3 and 4 contain only partial Piwi domain sequences (Figure 3.5A, C). However, no MID region between the PAZ and Piwi domains was detected in candidate 2 (Figure 3.4A). An aspartate/arginine (RD)-rich, as well as an arginine/glycine-rich (RGG domain) region towards the N-terminus of candidate 1 was also observed (Figure 3.3B). Candidate 1 shared significant similarity with *H. sapiens* (Q8TC59.1; 24% identity, 6^{e-26}) and *Mus musculus* (Q8CDG1.2 - 24% identity, 5^{e-25}) Ago 2 (Figure 3.3C), therefore, the protein has been named *Np*AGO-2. Candidate 2, on the other hand, is acknowledged as *Np*Piwil-2, due to its significant identity score (24 and 23%) against *Daphnia pulex* (EFX83175.1; 2^{e-14}) and *Xenopus tropicalis* (A8KBF3.1; 5^{e-12}) Piwi-like 2 homologues (Fig. 3b). Candidate 3, despite short in length, showed considerably identity homology (36-42% identity, e-value < 1e-5) with several Trypanosomatidae's Piwi-like1 proteins (CCC51171.1; CCC93424.1; ADI72731.1; EKG02139.1) (Figure 3.5B). These proteins are known as Piwi "solos" (PiwiS) as they lack the PAZ domain [56]. Therefore, candidate 3 has been regarded as *Np*-PiwiS. The remaining candidate shared 59% and 52% identity with *Gallus gallus* (A6N7Y9.1; 3^{e-07}, from 816-849 aa) and *Danio rerio*

(Q8UVX0.1; 1^{e-07}, from 797-840 aa) Piwi-like 1 isoform, respectively (Figure 5D), and has been referred as *Np*Piwil-1.

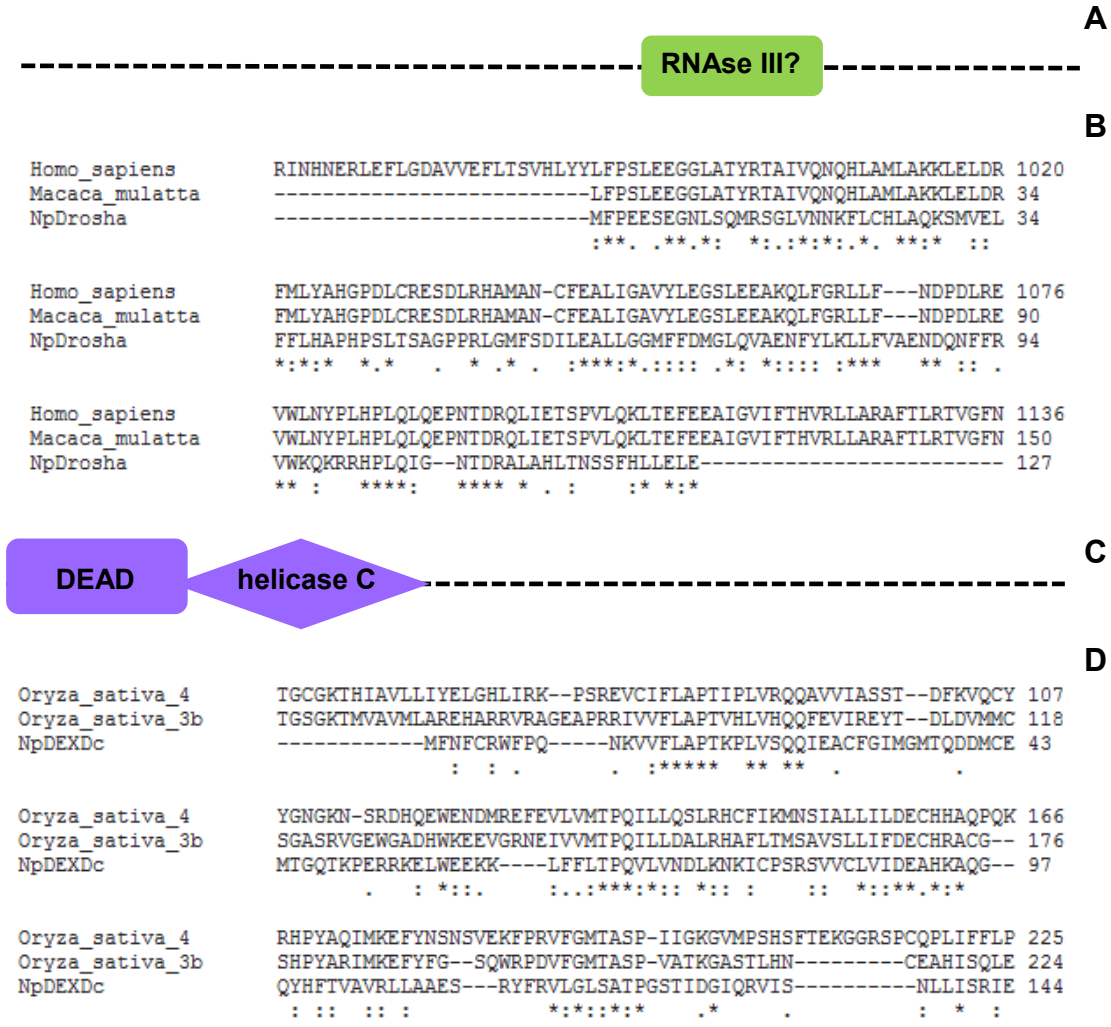

Figure 3.2 Candidates sharing significant level of homology with conserved motifs found in Dicer. (A and C) Schematic representation of the open reading frames containing RNAse III (*Np*Drosha) and DEXDc helicase (*Np*DEXDc) domains, respectively. Dotted lines indicate regions of unknown sequences. (B and D) Multiple sequence alignment of amino acid queries and their corresponding best-scoring hits. The bottom rows indicate the degree of conservation seen in the alignment column: (*) strictly conserved, (:) highly conserved or (.) moderately conserved.

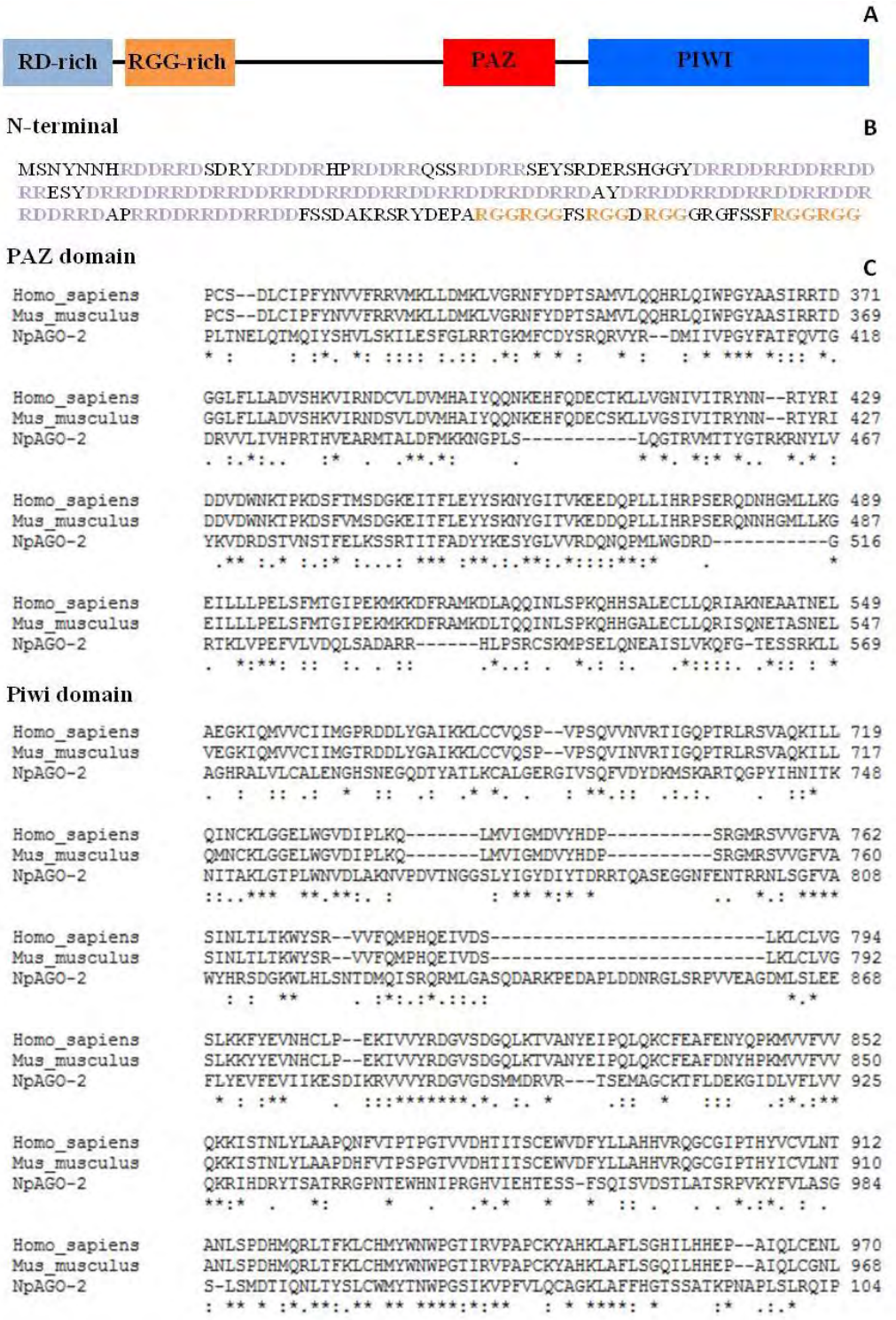

Figure 3.3 Candidate sharing high levels of homology with proteins from the Argonaute subfamily. (A) Schematic representation of detected conserved domains found in *Np*AGO-2. Solid lines indicate sequences with no recognizable domains. (B) Amino acid sequence corresponding to the N-terminal region. Purple and orange text indicates RD and RGG-rich regions, respectively. (C) Multiple sequence alignment between identified conserved domains (PAZ and Piwi) and their corresponding best-scoring hits. The bottom rows indicate the degree of conservation seen in the alignment column: (*) strictly conserved, (:) highly conserved or (.) moderately conserved.

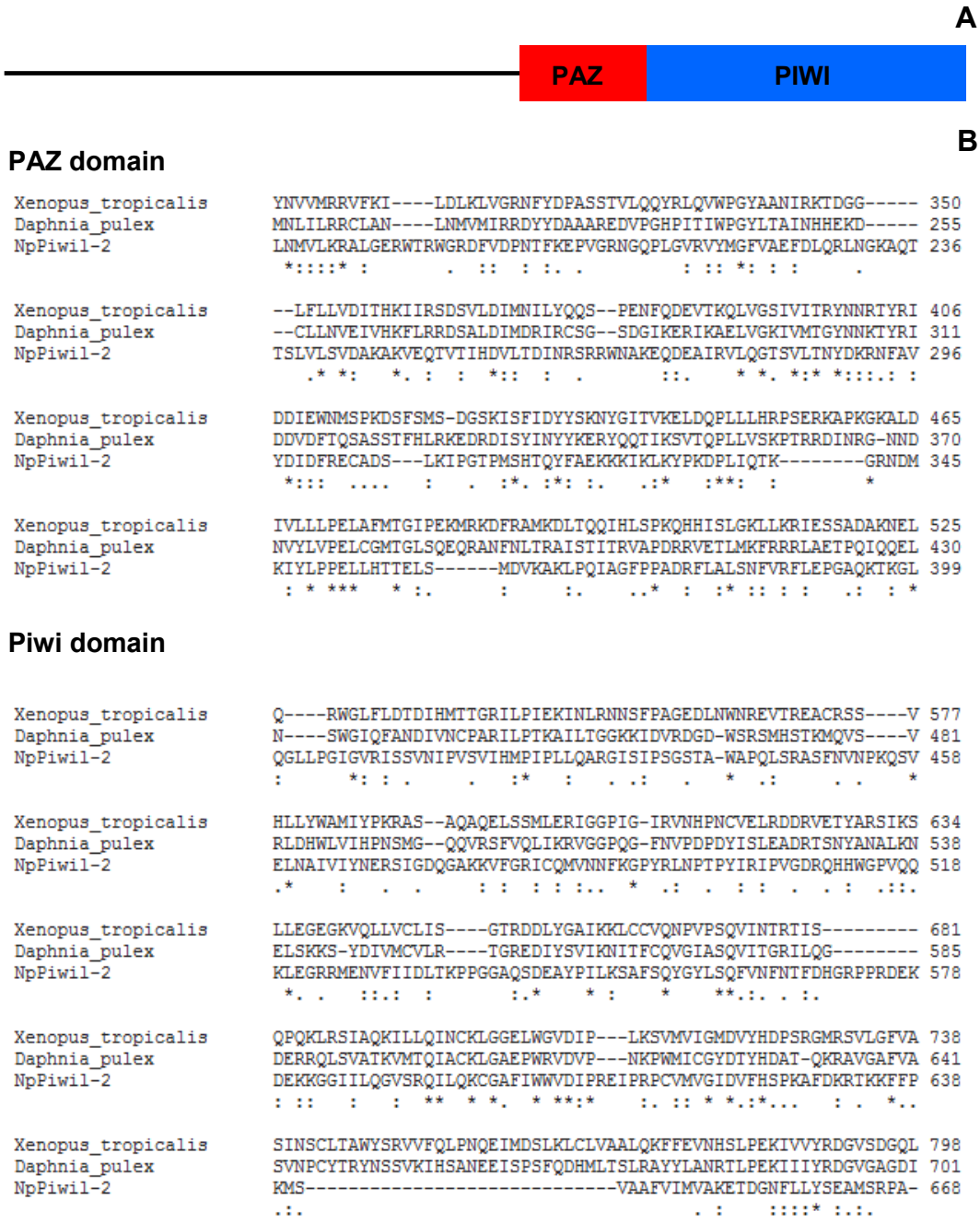

Figure 3.4 Candidate sharing high levels of homology with proteins from the Piwi subfamily. (A) Schematic representation of detected conserved domains found in *Np*Piwil-2. Solid lines indicate sequences with no recognizable domains. (B) Multiple sequence alignment between identified conserved domains (PAZ and Piwi) and their corresponding best-scoring hits. The bottom rows indicate the degree of conservation seen in the alignment column: (*) strictly conserved, (:) highly conserved or (.) moderately conserved.

Figure 3.5 Candidates partially covering the Piwi domain. (A and C) Schematic representation of open reading frames containing the Piwi domain. Dotted lines indicate regions of unknown sequences. (B and D) Multiple sequence alignment of amino acid queries and their corresponding best-scoring hits. The bottom rows indicate the degree of conservation seen in the alignment column: (*) strictly conserved, (:) highly conserved or (.) moderately conserved.

3.3.2. dsRNA and eiRNA validation

Long dsRNA targeting *N. pemaquidensis* β-actin and EF1-α, as well as non-specific luciferase, were effectively synthesized by *in vitro* transcription. Prominent bands of approximately the expected size were visualized on a 1.5% agarose gel (Figure 3.6, lane A). Gel electrophoresis also confirmed successful generation of esiRNA pools by RNAse III digestion of corresponding dsRNAs (Figure 3.6, lane B).

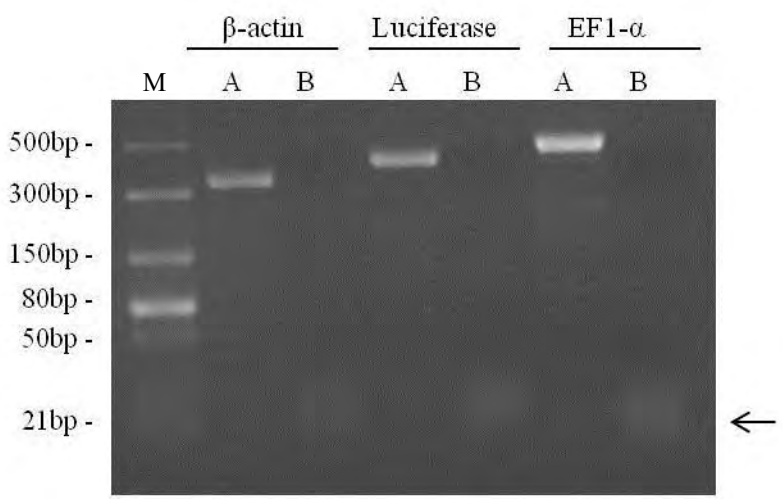

Figure 3.6 Validation of dsRNA and esiRNA synthesis by gel electrophoresis. (A) dsRNA corresponding to *N. pemaquidensis* β-actin and EF1-α, as well as luciferase, were synthesised by *in vitro* transcription using MEGAscript® T7 Kit. (B) Following transcription, a heterogeneous mix of siRNAs from each dsRNA was prepared by RNAse III digestion (esiRNAs). Approximately 500 ng of dsRNA was loaded in each lane of a 1 x TBE 1.5% agarose gel. (→) Indicates cleaved product (~ 20 bp esiRNAs). (M) dsRNA marker (21-500 bp).

3.3.3. RNAi duplexes up-take

In order to confirm if RNAi duplexes were incorporated by the amoeba through phagocytosis, a fluorescent siRNA control was delivered into the culture in the presence and absence of transfection reagent. As demonstrated by microscopy analysis, strong fluorescent signal was detected in transfected trophozoites which exhibited fluorescent food vacuoles and diffuse staining in the cytoplasm (Figure 3.7A). Although significantly weaker, some fluorescent signal was also observed in amoeba soaked with fluorescent siRNA alone (Figure 3.7B). The results suggest that RNAi duplexes are released into the amoeba cytoplasm even in the absence of transfection reagent, albeit not as efficiently.

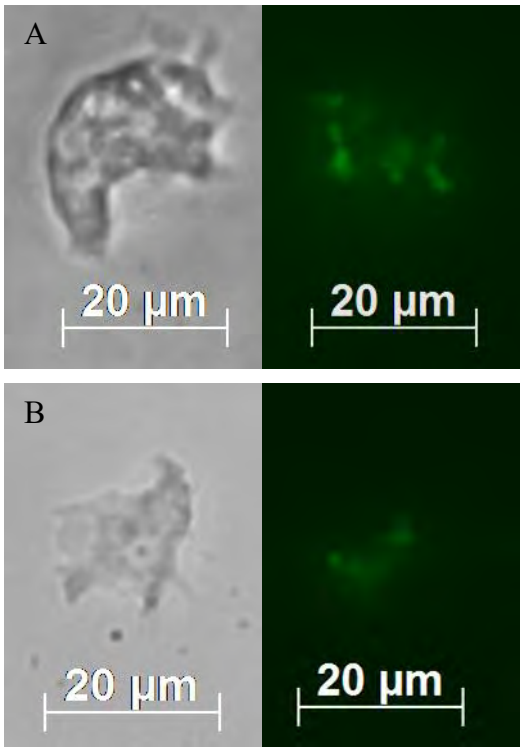

Figure 3.7 Validation of RNAi duplexes up-take by the amoeba. The figures show phase contrast and fluorescent image of the same trophozoite after 24 hours incubation with fluorescent siRNA control in the presence (A) or absence (B) of transfection reagent. (40x objective, scale bar 20 µm).

3.3.4. Dicer and Argonaute up-regulation

Activation of the *N. pemaquidensis* RNAi pathway in the presence of RNAi duplexes was assessed by qRT-PCR, 24 h post dsRNA and esiRNA administration. Relative quantification of gene expression revealed that *Np*DEXDc was significantly up-regulated by approximately 2.4 ±0.5 fold due to dsRNA delivery (Figure 3.8). *Np*Drosha mRNA levels, on the other hand, were not significantly influenced by the presence of dsRNA. For Ago candidates, only *Np*AGO-2 mRNA transcripts showed significant up-regulation of 3.55 ±1.02 and 7.22 ±0.31 fold when the esiRNA transfection mix was incorporated into the amoeba culture containing or lacking lipofectamine, respectively (Figure 3.8).

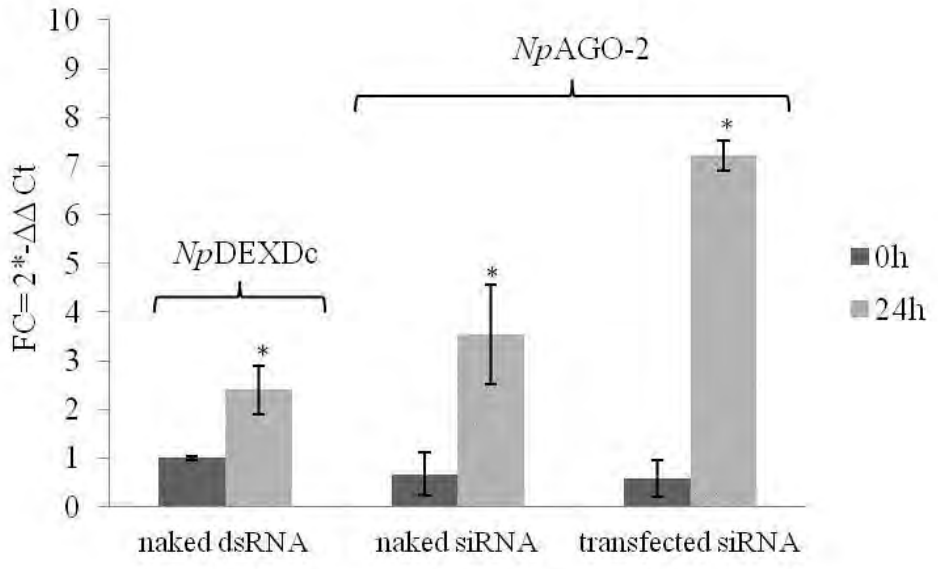

Figure 3.8 Up-regulation of Dicer (*Np*DEXDc) and Argonaute (*Np*AGO-2) candidates following the administration of dsRNA and esiRNA, respectively. Bars symbolize the mean value ±S.E. (n= 4) at each sampling period and asterisks "*" indicate significant difference between time points.

3.3.5. Gene silencing by in vitro transcribed dsRNA

In vitro transcribed dsRNAs targeting fragments of *N. pemaquidensis* specific genes (β-actin and EF1-α), as well as luciferase, were synthesised using MEGAscript® T7 kit (Invitrogen) and administered to a final concentration of 20 µg/mL into the amoeba culture. Relative quantitation by comparative Ct method revealed that β-actin and EF1-α mRNA levels were decreased by approximately 47.6 ±23.6% (Figure 3.9A) and 37 ±17.8% (Figure 3.9C), respectively. However, due to high variation across replicates, the observed reductions were not significantly different from luciferase-dsRNA treatment. Interestingly, gel electrophoresis showed a considerably fainter band in the PCR reaction containing β-actin specific primers and pooled cDNA samples from β-actin-dsRNA treated group at 24 h (Figure 3.9B).

No significant variation of the expression level for either target genes was observed by the administration of the non-specific control (luciferase-dsRNA).

3.3.6. Gene silencing by esiRNA pool

esiRNA pools generated by RNAse III digestion of the target dsRNAs were delivered to the amoeba culture at 10 nM, in either the presence or absence of transfection reagent. When β-actin-esiRNAs were directly incorporated into the amoeba culture, β-actin relative expression levels showed similar trend to when the corresponding dsRNA was administrated, i.e. a subtle reduction at 24 h (Figure 3.9A). However, when trophozoites were transfected with the same siRNA duplexes, β-actin mRNA transcripts were significantly knocked down by 66 ±10% at 24 h (Figure 3.9C). Surprisingly, the silencing effect was not significant for more than 24 h. While β-actin was successfully downregulated only with the aid of lipofectamine, EF1-α mRNA levels were significantly depleted at 72 h with both delivery strategies (Figure 3.10E, G). Nevertheless, transfected amoeba presented higher silencing efficiency (93 ±5.25%) (Figure 3.10G) as opposed to those treated with esiRNA transfection mix lacking lipofectamine (57±15.5%) (Figure 3.10E). Expression levels of β-actin and EF1-α were again not significantly affected by the presence of the control treatment.

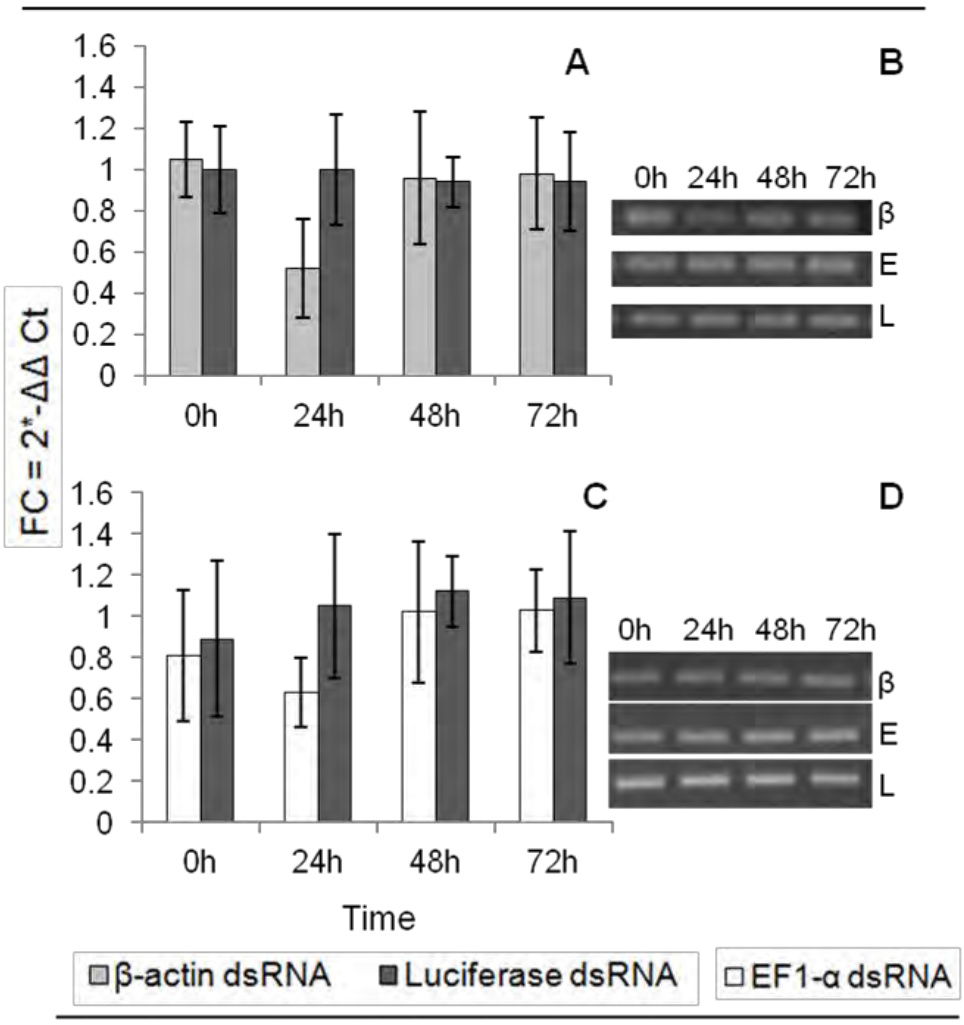

Figure 3.9 qRT-PCR analysis of β-actin and EF1α relative expression levels in amoebae treated with *in vitro* transcribed dsRNA via immersion (a, c). Bars symbolize the mean value ±S.E. (n= 4) and asterisks "*" indicate significant difference between time points. (b, d,) are representative gels of RT-PCR products of β-actin and EF1α gene expression at each sampling period. Row β – amoebae treated with β-actin-dsRNA; Row E – amoebae treated with EF1α-dsRNA; Row L - amoebae treated with luciferase-dsRNA.

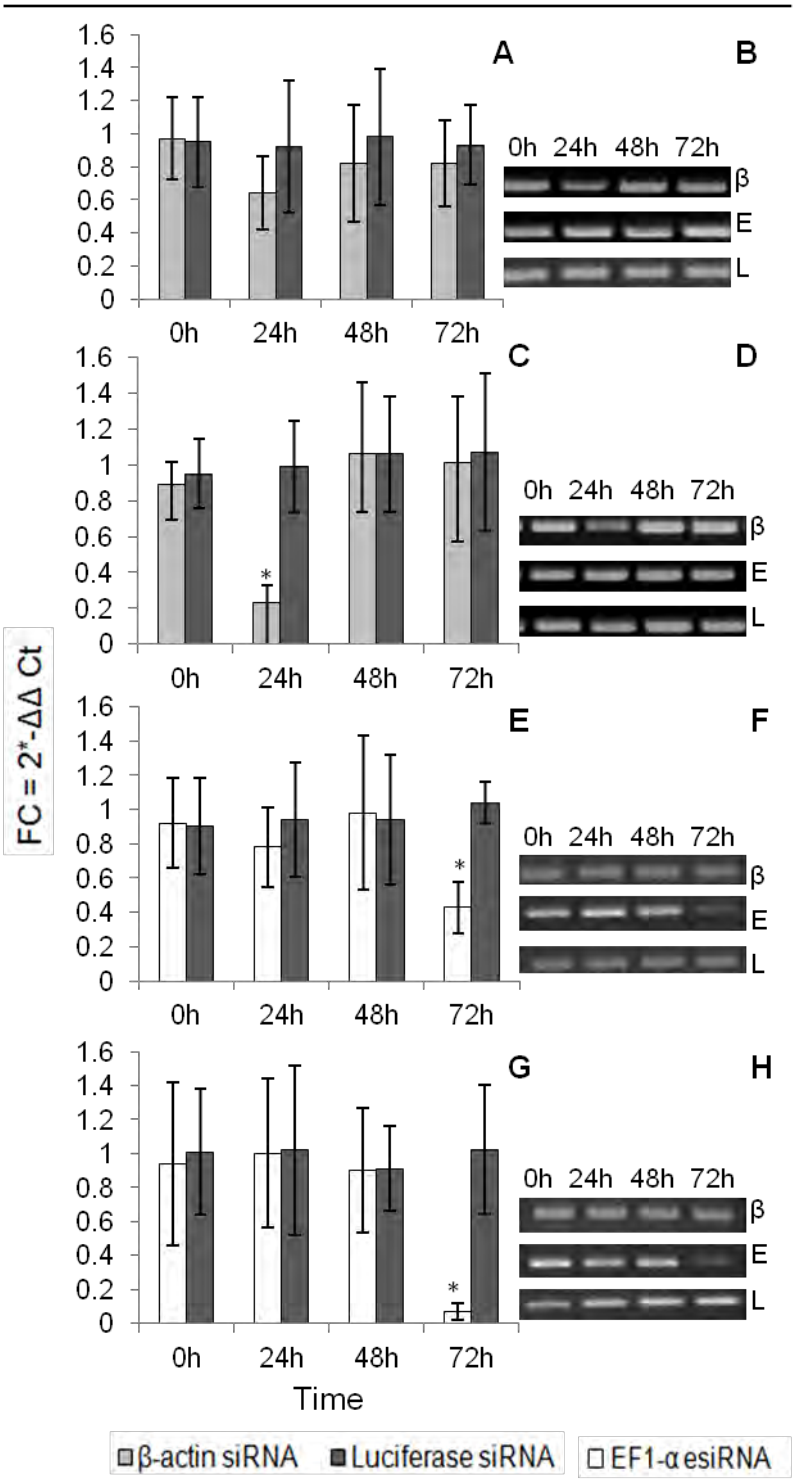

Figure 3.10 qRT-PCR analysis of β-actin and EF1α relative expression levels in amoebae treated with esiRNAs via immersion (A, E) **and transfection** (C, G). Bars symbolize the mean value ±S.E. (n= 4) and asterisks "*" indicate significant difference between time points. (B, D, F, H) are representative gels of RT-PCR products of β-actin and EF1α gene expression at each sampling period. Row β – amoebae treated with β-actin-esiRNA; Row E – amoebae treated with EF1α-esiRNA; Row L - amoebae treated with luciferase-esiRNA.

3.3.7. RNAse III activity

The ability of *N. pemaquidensis* Dicer to cleave long dsRNA was validated by incubating *in vitro* transcribed dsRNA in the presence of amoeba lysate. Gel electrophoresis analysis of incubated dsRNA showed complete degradation of the RNA duplexes, as earlier as 1 h after continuous incubation (Figure 3.11, lane C). As expected, the digestion efficiency was significantly reduced when serially diluted lysates were added to the substrate (Figure 3.11, lanes D-H). Additionally, the dsRNA remained intact when the lysate was treated with 10x EDTA, suggesting inactivation of Dicer enzymatic activity (Figure 3.11, lane I).

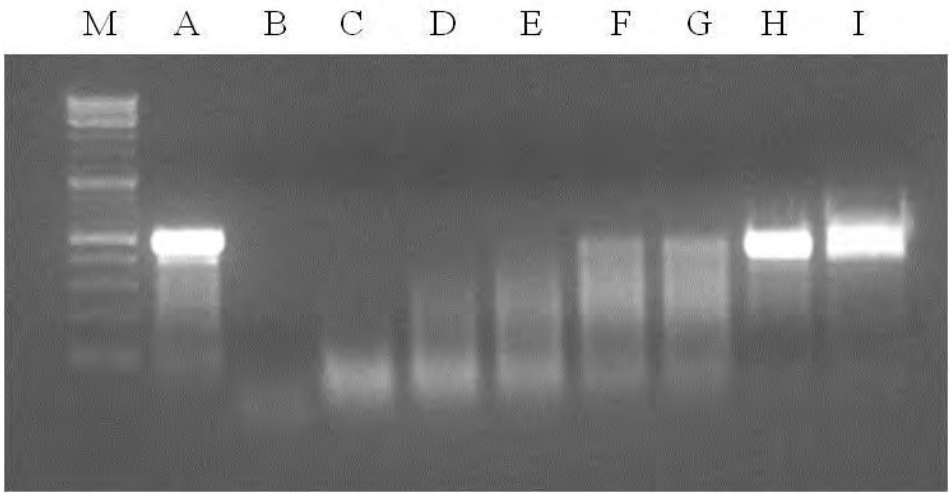

Figure 3.11 Agarose gel demonstrating the RNAse III activity assay. The presence of an active Dicer was supported by analysing dsRNA cleavage following incubation with *N. pemaquidensis* lysate. (A) 2 µg of dsRNA, (B) 2 µg of dsRNA incubated with commercially available *E. coli* RNAse III, (C-G) 2 µg dsRNA incubated with serially diluted *N. pemaquidensis* lysate (1:50, starting at 5 µg), (H), 2 µg of dsRNA incubated with buffer without amoeba lysate, (I) 2 µg of dsRNA incubated with *N. pemaquidensis* lysate (5 µg), previously inactivated with 10x EDTA. All treatments were incubated for 1 h. (M) 2-Log DNA marker (0.1-10 kb).

3.4. Discussion

RNAi has been proposed as an evolutionary conserved mechanism in plant and animal cells. Nevertheless, in the case of protozoan parasites, which are primitive eukaryotes, data from a variety of studies has led to the conclusion that RNAi has failed to silence genes in certain species [286, 287] or, in other cases, RNAi was highly gene-specific [62, 288]. In the present study we employed functional and comparative genomic approaches to validate the presence of functional RNAi machinery in a member of the *Neoparamoeba* genus. Analysis of a *N. pemaquidensis* transcriptome database revealed the presence mRNA encoding proteins homologous to those described as being involved in homology-dependent gene silencing.

A definitive Dicer homologue was not confirmed in *N. pemaquidensis*, however conserved domains commonly found in Dicer proteins of higher eukaryotes were indentified. Two partial protein sequences shared significant similarity to Dicer DEXDc DEAD-like helicase (*Np*DEXDc) and RNAse III (*Np*Drosha) conserved domains. However, while *Np*DEXDc showed high level of homology with Dicer proteins, the other candidate (*Np*Drosha) was highly related with Drosha, which is known for containing two RNase III domains and a dsRBD at the C-terminal end (Figure 1B) [289]. Drosha is a RNAse III protein responsible for cleaving pri-miRNAs to yield hairpin-shaped pre-miRNAs for further processing by the cytoplasmic RNAse III enzyme, Dicer [94]. qRT-PCR analysis confirmed these findings demonstrating that *Np*DEXDc, but not *Np*Drosha, was significantly up-regulated in the presence of *in vitro* transcribed dsRNA, suggesting the presence of both siRNA and miRNA pathways. Interestingly, the only *Entamoeba histolytica* Dicer candidate (EAL45114) also contains just an RNAse III domain. However, in

contrast to what was seen in our study, this has shown to be functionally involved in dsRNA cleavage [285]. Pairwise alignment of both *N. pemaquidensis* and *E. histolytica* RNAse III-containing sequences revealed that these proteins are not significantly similar (21% identity, E-value = 0.069) to one another, providing further indication that *Np*Drosha is not involved in the classic RNAi pathway.

Strong evidence for the presence of an active Dicer in *N. pemaquidensis* was also supported by the RNAse III activity assay. The results showed complete dsRNA digestion following incubation with amoeba lysate, as well as inactivation of Dicer enzymatic activity by the addition of 10x EDTA. However, despite the above findings, administration of *in vitro* transcribed dsRNA targeting *N. pemaquidensis* β-actin and EF1-α failed to elicit significant knockdown of either target genes, when delivered via immersion at 20 µg/mL. A non significant slight reduction of β-actin (47.6 ±23.6%) and EF1-α (37 ±17.8%) mRNA levels was detected at 24 h (data supported by electrophoresis). However, we believe the observed downregulation could be enhanced by either administration of higher dsRNA doses or through direct transfection. For example, Solis, Santi-Rocca [61] demonstrated that successful knockdown of *Entamoeba histolytica* KERP-1 protein was triggered when amoebae were directly soaked with bacterially expressed dsRNA at 50 µg/mL, but not with lower dosages (2 and 25 µg/mL). Chen, Yang [290], on the other hand, used transfection to show that dsRNA doses as low as 5 µg/mL were sufficient to significantly silence the parasite *Trichinella spiralis* target gene. Conversely, using a considerably low dsRNA concentration (0.2 µg/mL), Barry, Alberdi [291] revealed that *in vitro* transcribed dsRNA administered either in the presence or absence of transfection reagent could effectively silence the target gene in several tick cell lines. The selected dsRNA concentration used in the current experiment was based on previous studies performed using bacterially expressed dsRNA (Chapter 4).

Therefore, further optimization is required to ensure that *in vitro* transcribed dsRNA can elicit successful silencing effect in *N. pemaquidensis* as dsRNA-mediated knockdown can be highly affected by the target organism, delivery system, RNAi duplex and dosage.

Four protein candidates encoded conserved regions of the Ago superfamily: two with homology to both PAZ and Piwi domains (*Np*AGO-2 and *Np*Piwil-2) and two partial open reading frames sharing significant homology with the Piwi domain (*Np*-PiwiS and *Np*Piwil-1). Similarly to the *T. brucei* Ago (*Tb*Ago1), *Np*AGO-2 presented a N-terminal domain with a high abundance of RGG repeats. According to Shi, Chamond [292], this RGG-repeat domain is essential for the association of *Tb*Ago1 with polyribosomes and, consequently, functionally relevant for RNAi-mediated degradation of mRNA. Therefore, considering the presence of a RGG-rich region, together with our candidate sharing significant homology with AGO-2 from several species, we propose that *Np*AGO-2 is involved in siRNA-mediated mRNA degradation. However, unlike *Tb*Ago1, a longer aspartate-arginine-rich region was also observed in the N-terminal portion of *Np*AGO-2 which, to our knowledge, has not been previously reported in Ago proteins.

The involvement of *Np*AGO-2 in the canonical RNAi pathway was reinforced by qRT-PCR analysis, where a significant increase in *Np*AGO-2 mRNA levels following the administration of esiRNAs, either in the presence or absence of lipofectamine was observed. While *Np*AGO-2 was highly related to proteins from the Ago subfamily, *Np*Piwil-1 and 2 presented significant identity scores against members of the Piwi clade. However, unlike *Np*Piwil-2, which covers both PAZ and Piwi conserved domains, *Np*Piwil-1 contains only a partial segment of the Piwi motif. Unsurprisingly, the expression levels of neither candidate were influenced by the presence of esiRNAs, suggesting that these proteins are not involved in the

siRNA-mediated silencing pathway. Therefore, the results suggest that *N. pemaquidensis* retained key genes involved not only in the RNAi machinery, but also in the miRNA and piRNA pathways. A fourth putative protein (*Np*PiwiS) partially encoding the Piwi domain shared significant similarity to the Trypanosomatids PiwiS, which are known for lacking a recognizable PAZ domain. However, since there is still no evidence that these could carry out functions related to any of the small RNA-mediated pathways, the biological role of these proteins remains unknown, although it has been suggested to represent a member of a novel Ago subfamily [293].

Functional evidence for an active RNAi machinery was also supported by esiRNA-mediated gene silencing. *N. pemaquidensis* treated with either naked or transfected EF1-α esiRNAs, at 10 nM, presented a significant reduction of the target gene transcript levels at 72h. Furthermore, the use of lipofectamine significantly improved the observed silencing efficiency by approximately 36%. The results were consistent with what was seen when the fluorescent siRNA control was administered, i.e. transfected trophozoites displayed considerably stronger fluorescent signal than the ones simply soaked with the same RNAi duplexes. The data above suggests that the effectiveness of EF1- α knockdown was directly associated with the amount of esiRNAs available in the amoeba cytoplasm. In contrast, β-actin mRNA levels were significantly depleted only when esiRNAs were delivered by transfection. However, similarly to what was observed when amoebae were soaked with *in vitro* transcribed dsRNA against the same target gene, a small but not significant reduction of β-actin expression levels was observed at 24 h, when esiRNAs were added directly into the culture. Therefore, considering β-actin is a very abundant cellular protein and also that amoeba trophozoites are constantly replicating by meiosis, we believe that higher amounts of RNAi duplexes are required to enter the RNAi pathway in order to

achieve successful knockdown of such gene. Unfortunately, we were unable to validate suppression at the protein level, as there are no *N. pemaquidensis* antibodies to β-actin and EF1-α available at the present time.

In conclusion, we employed comparative genomic approaches to identify key components of the RNAi pathway from the *N. pemaquidensis* transcriptome. BLAST analysis revealed translated protein sequences containing conserved domains found in Dicer and Ago. Apart from sharing significant homology against the corresponding proteins from other species, we have provided evidence that *Np*DEXDc and *Np*AGO-2 were significantly up-regulated when amoebae were administered with dsRNA and esiRNA, respectively, indicating their involvement in the siRNA-mediated silencing pathway. The presence of a functional RNAi machinery was also supported by the gene silencing assay, which showed that esiRNA targeting *N. pemaquidensis* β-actin and EF1-α resulted in specific knockdown of target transcripts. Transcriptome screening also revealed partial amino acid sequences containing conserved motifs of Drosha and Piki-like proteins, suggesting that miRNA and piRNA may also be present in *N. pemaquidensis*. However, efforts still need to be directed towards obtaining the full sequence of the ORFs of interest.

The results altogether provide strong evidence for the presence of an active RNAi machinery in *N. pemaquidensis*. While the outcomes are promising, further studies need to be carried out to validate if RNAi is evolutionarily retained in the closely related species *N. perurans*. Such finding would open up the possibility of employing RNAi as a research tool to assist in the development of new treatment strategies against AGD. In this context, the Atlantic salmon industry could greatly benefit from such technology, as unravelling the molecular complexity of amoeba biology, as well as the relationship between host and parasite, are crucial for the

discovery of novel intervention strategies and identification of new treatment candidates.

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CHAPTER 4: Experiment - 2

First evidence of functional RNAi mechanism in *Neoparamoeba* genus

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Abstract

RNA interference (RNAi) has been extensively used to study gene function in non-model organisms and has the potential to identify parasite target molecules in order to develop alternative treatment strategies. This technology could assist in further development of preventive methods against amoebic gill disease (AGD), the main health problem affecting the Atlantic salmon aquaculture industry in Tasmania, Australia, and now a significant emerging issue in Europe. Using β-actin and EF1-α as candidate genes, we investigated the feasibility of gene knockdown by double stranded RNA (dsRNA) in *Neoparamoeba pemaquidensis*, the non infective strain closely related to the causative agent of AGD, *Neoparamoeba perurans*. Bacterially expressed dsRNA targeting the selected target genes was administered by soaking (2, 20 and 50 µg/mL) and a time course sampling regime performed. Quantitative real time PCR analysis showed that candidate genes were successfully downregulated with silencing efficiency and duration both target and dose-dependent. Additionally,

β-actin deficient trophozoites unexpectedly transformed into a cyst-like stage, which has not been previously reported in this species. These results altogether are the first evidence that functional RNAi machinery is present in the genus *Neoparamoeba*.

Keywords: amoeba; aquaculture; salmon; RNA interference; soaking

4.1. Introduction

Amoebic gill disease (AGD), caused by the amphizoic amoebae *Neoparamoeba perurans*, is considered the major health concern affecting marine Atlantic salmon aquaculture in Tasmania, Australia [36]. At the moment, bathing the fish in freshwater is the only commercially effective treatment available [21]. However, since this practice was first introduced in the late 1980s, the bathing frequency throughout the marine production cycle has tripled in order to successfully avoid AGD progression during the same period [18]. Therefore, due to the high costs associated with treatment and lost productivity, as well as limited freshwater resources in Tasmania, bathing is not considered a viable long-term management solution against AGD [281]. As a result, the development of improved therapeutical strategies for coping with this disease is imperative for the continued sustainability of the Tasmanian Atlantic salmon aquaculture industry.

One recent technology likely to play a major role in the future of aquaculture is RNA interference (RNAi). RNAi is a highly conserved mechanism of post-transcriptional gene silencing in which double-stranded RNA (dsRNA) corresponding to a gene, or coding region, of interest is introduced into an organism, resulting in degradation of the corresponding mRNA [75]. Because of this sequence-specific ability to silence target genes, RNAi has greatly facilitated the analysis of gene function, especially in non-model organisms that are not amenable to classical genetic approaches [283]. With regard to parasitic diseases of farmed fish, the analysis of gene function through RNAi could be used not only to investigate the interaction between host and parasite, but also to explore the parasite biology and the effects of knockdown on its survival. This could assist in the identification and validation of new anti-parasitic treatment candidates.

Since its initial discovery, the RNAi silencing mechanism has been found to be widely conserved in a variety of eukaryotic systems, ranging from unicellular protozoans, insects and fungi to complex organisms such plants and vertebrates [85, 86, 88, 294-296]. However, many studies have shown that the RNAi machinery appears to have been repeatedly lost in certain protozoan parasites [53]. Surprisingly, this occurrence is not explained by parasite phylogeny [297]. For example, whereas there is strong functional and genomic evidence for the presence of an active RNAi pathway in *Trypanosoma brucei* and *T. congolense* [57-60], other members of the same family such as *T. cruzi*, *Leishmania major* and *L. donovani*, are known to be RNAi negative [55, 56]. Numerous hypotheses attempting to elucidate such occurrences have been proposed in many studies [53, 56, 297]. However, the apparent absence of a functional RNAi pathway in certain eukaryotic microbes is still a puzzle. Therefore, before the RNAi technology can be explored as a tool to assist in efforts to control AGD, functional and comparative genomic studies should be undertaken to confirm the presence or lack of an active RNAi pathway in *N. perurans*. As the *in vitro* culture of *N. perurans* only recently became available [36], the aim of the present study was to provide experimental evidence for the presence of functional RNAi machinery in the closely related *Neoparamoeba pemaquidensis*, a non-infective strain of the genus *Neoparamoeba.* Using *N. pemaquidensis* β-actin and EF1-α as target genes, we aimed to evaluate the feasibility of gene knockdown by the administration of bacterially expressed double stranded RNA (dsRNA) via soaking.

4.2. Material and Methods

4.2.1. *Neoparamoeba pemaquidensis* culture conditions

N. pemaquidensis, the non-infective strain of the *Neoparamoeba* genus, was used in the present experiment. For this purpose, *N. pemaquidensis,* originally isolated and cloned from AGD affected salmon, were grown on malt-yeast-seawater agar plates and kept at 16°C. Prior to each experiment, the agar plates were rinsed with sterile seawater and the amoeba suspension transferred to a fresh falcon tube. Following centrifugation, the amoeba pellet was washed to reduce bacterial load and resuspended in sterile seawater. The amoebae cells were quantified using a haemocytometer and equally transferred to culture plates to a final concentration of 10^5amoebae/mL/well. Plates were incubated at 16°C until the end of each experiment.

4.2.2. RNA extraction and reverse transcription

Total RNA was isolated from *N. pemaquidensis* cells using Trizol® (Invitrogen), according to the manufacturer's instructions. RNA was quantified using a NanoDrop ND-1000 Spectrophotometer and its integrity assessed on 1.5% TAE agarose gel. RNA was subsequently treated with TURBO DNA-free™ (Ambion) to remove any contaminating DNA and reverse transcribed using Superscript™ III Reverse Transcriptase with oligo(dT) primer (Invitrogen).

4.2.3. Synthesis of bacterially expressed dsRNA

For the construction of dsRNA-expression vectors, specific primers designed against the full coding sequences of *N. pemaquidensis* β-actin and EF1-α, as well as luciferase, were used to amplify fragments of 500, 700 and 600 bp, respectively (Table 4.1) (see Appendix for sequences).

Table 4.1 Primer sequences used for construction of dsRNA-expression vectors (ds) and qRT-PCR (q).

primer	nucleotide sequences (5'-3')
ds_β-actin.5'	GATACTAGTACCTTCAACACCCCCGCCATG
ds_β-actin.3'	AACGCTAGCTAGGACTTCTCGAGGGCAGAG
q_β-actin.5'	CAATCCAAGCGTGGTATCCT
q_β-actin.3'	GCTCGTTGTAGAAGGTGTGG
ds_EF1α.5'	GATACTAGTGCCGGAAAGTCCACCACCACT
ds_EF1α.3'	AACGCTAGCTGTACCCGATCTTCTTCAAGA
q_EF1α.5'	GTACAAGGGTCCCACTCTCCC
q_EF1α.3'	AGCGAAGGTGACAACCATAC
ds_ludiferase.5'	GATACTAGTATGGAAGACGCCAAAAACATA
ds_luciferase.3'	AACGCTAGCAACCCCTTTTTGGAAACAAAC

The amoeba sequences were obtained from sequence analysis of a normalized EST library (data not published). Following PCR amplification using Phusion DNA polymerase (Invitrogen), the fragments were cloned into pAcquire vector and the purified plasmids submitted to restriction enzyme digestion with Nhe I and Spe I (New England BioLabs). The correct sized bands were then gel purified and subcloned into the double T7 promoter vector PL4440. Following cloning, the nucleotide sequences of the recombinant plasmids were confirmed by DNA sequencing and the resultant constructs transformed into HT115 (DE3) RNase III-deficient *E. coli* strain, which is modified to express T7 RNA polymerase from an

IPTG-inducible promoter. In addition to luciferase, an empty L4440 vector without any insert was also transformed into HT115 (DE3) and used as negative control.

4.2.4. Bacterial IPTG induction

Single colonies of HT115 (DE3) bacteria containing cloned L4440 plasmids were grown in LB medium containing 12.5 µg/mL tetracycline and 100 µg/mL ampicillin at 37 °C, with shaking. Overnight bacterial cultures were diluted 50-fold in 2YT medium containing the same antibiotics and cultured at 37 °C to an optical density of 0.6 at 600 nm. The expression of dsRNA was then induced by the addition of isopropyl-β-D-thiogalactopyranosine (IPTG) to a final concentration of 2 mM, followed by additional 4 h incubation at 37 °C. The bacterial cells were harvested by centrifugation at 6000 x g for 10 min at 4 °C.

4.2.5. dsRNA purification from bacteria

The dsRNA was purified from the bacteria using a protocol adapted from Ongvarrasopone et al. [264] and Solis et al. [61]. Briefly, following centrifugation, every 1 mL of bacterial pellet was resuspended in 50 µL of 0.1% SDS and boiled for 2 min to lyse the cells. Total RNA was isolated from the bacterial lysate using Trizol® (Invitrogen), and subsequently incubated for 1h at 37°C with 0.4 U/µL of Turbo DNase and 0.2 µg/µL of RNase A (Ambion) to remove contaminating genomic DNA and single-stranded RNA, respectively. The remaining dsRNA was then purified with an equal volume of phenol:chloroform:isoamyl alcohol (Sigma) and precipitated with 0.5 volume of 7.5 M ammonium acetate and 1 volume of isopropanol. Following centrifugation at 12,000 g for 30 min, the dsRNA-containing

pellet was washed twice with ethanol 70% and resuspended in nuclease free water. Double-stranded RNAs were analysed by agarose gel electrophoresis and concentration determined using a NanoDrop ND-1000 Spectrophotometer. The dsRNA integrity was further confirmed by ShortCut RNAse III (NEB) digestion at 37 °C for 20 min.

4.2.6. Delivery of bacterial dsRNA via soaking

Prior to the experiments, the amoebae were transferred to 24-well culture plates (Nunclon^{TM} Delta Surface) containing 1 mL of filtered seawater in each well. The treatments were performed in quadruplicate and each replicate comprised all the amoebae within a single well (10^5 amoebae). Purified dsRNA targeting the *N. pemaquidensis* β-actin and EF1-α, as well as the unrelated luciferase and the empty L4440 vector, were directly administrated by immersion to a final concentration of 2, 20 and 50 µg/mL of culture media. When β-actin knockdown was being assessed, samples treated with dsRNA expressing EF1α were regard as internal controls and *vice versa*. dsRNA exposed amoebae were cultured under standard *in vitro* conditions and sampled at 0, 6, 12, 24, 48 and 72 h post dsRNA administration. A second experiment was also performed with the aim to assess whether continuous administration of dsRNA would result in more effective downregulation of the targeted genes as opposed to a one-off addition. For this purpose, daily administration of 20 µg/mL of each dsRNA construct was performed and sampling carried out at 0, 24, 48, 72 h and 7 days following the first dsRNA administration.

4.2.7. Total RNA extraction and RT-PCR

At every sampling time point, the seawater from the sampled wells was removed by pipetting and each well rinsed twice with filtered seawater to eliminate unattached debris and remaining dsRNA. The amoebae were then detached from the culture plates by adding Trizol® (Invitrogen) and transferred to fresh 1.5 mL tubes. Total RNA was isolated from dsRNA treated amoebae as described previously, followed by Turbo DNAse-treatment (Ambion) and reverse transcription (Invitrogen). To determine whether the β-actin and EF1-α transcripts were effectively silenced, RT-PCR was conducted using the synthesized cDNA as a template in a reaction containing GoTaq® (Promega) and the target genes' specific primers. The temperature profile for PCR amplification was performed by holding at 94 °C for 5 min, followed by 28 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min; and a final extension at 72 °C for 7 min. The PCR products were analysed by agarose gel electrophoresis and the mRNA expression of the target genes evaluated using the intensity of the bands from luciferase-dsRNA treated samples as a control.

4.2.8. Quantitative real time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed on an ABI 7600 system to determine whether the administration of bacterially expressed dsRNA targeting the *N. pemaquidensis* β-actin and EF1-α would significantly downregulate the mRNA expression levels of the target genes. Previous studies performed in our laboratory have shown that dsRNA submitted to reverse transcription can be detected during quantitative real time PCR (qRT-PCR) (results not shown). Therefore,

considering that dsRNA residues could remain attached to the amoebae membrane, qRT-PCR primers specific to each target gene (Table 1) were designed to amplify different sections of the gene sequence than those selected to build the dsRNA constructs. All reactions were performed in triplicate, each containing 4 μL of diluted cDNA, 2 x SensiMix SYBR (Bioline) and 0.5 mM forward and reverse primers in a 10 μL reaction. The amplification profile consisted of an initial denaturation step at 95 °C for 10 min; 40 cycles of 95 °C for 20 s, 58 °C for 20 s and 72 °C for 20 s, followed by a dissociation stage according to the manufacturer's instructions. Dissociation curve analysis was performed to verify the specificity of the PCR amplification. Absolute quantification of β-actin and EF1-α was determined by generating external standard curves using 10-fold serial dilution of plasmid DNA as templates. The absolute amount of each target gene was expressed as copy number using the following equation:

$$\textbf{Quantity} = 10^{(Ct-b)/m}$$

Where Ct is the threshold cycle, b is the y-intercept and m is the slope of the linear regression equation obtained from each standard curve. The efficiency of each primer set was calculated from the formula $E = 10^{-1/slope}$. The expression levels of β-actin and EF1-α were presented as relative copy number which was normalized against the samples that received empty L4440 vector treatment.

4.2.9. Statistical analysis

Differences in β-actin or EF1-α expression levels within treatments, at each sampling period, was calculated by one-way analysis of variance (ANOVA) using the R version 2.14 software (R Development Core Team 2007). Values were

considered to be significant at $p < 0.05$. All numerical data were expressed as the mean ± standard error.

4.2.10. Microscopy

Possible phenotypic changes in dsRNA-treated amoeba were examined by microscopy, using a Zeiss Axio Observer inverted microscope, equipped with Zeiss AxioCam CCD camera and AxioVision Software (Carl Zeiss, Jena, Germany). Aliquots of trophozoites soaked for 72 h in 50 μg/mL of each treatment were transferred to glass slides carefully covered with a coverslip and immediately visualised under 10x and 40x magnification. Oil immersion was employed when the 100x objective was used. The 50 μg/mL dose was selected as it efficiently silenced both target genes.

4.3. Results

4.3.1. Verification of dsRNA integrity

Long dsRNA targeting both *N. pemaquidensis* target genes (β-actin and EF1-α), as well as the unrelated luciferase and the empty L4440 vector, were successfully produced in RNase III deficient *E. coli*. As expected, a small product of approximately 220 bp was detected in samples extracted from bacteria transformed with L4440 vector only (Figure 4.1), presumably representing the uncut multiple cloning site (MCS) of this vector.

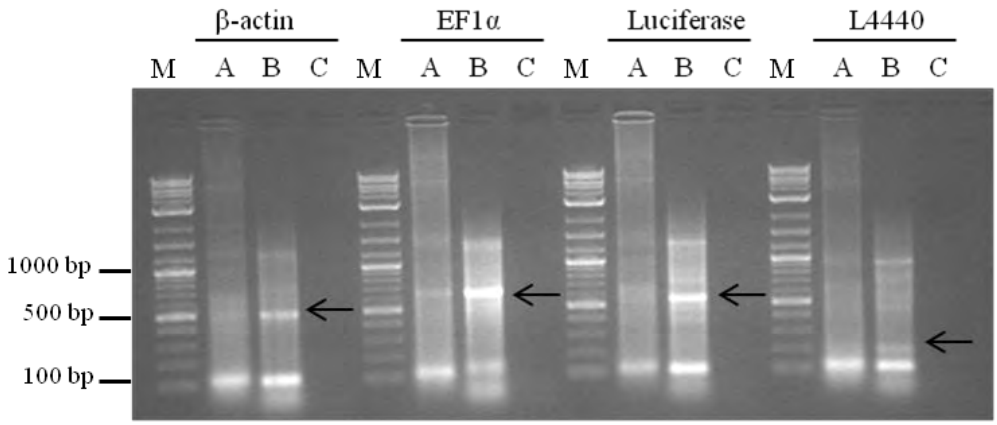

Figure 4.1 Agarose gel demonstrating dsRNA construct integrity. Following TRIZOL® extraction (A) each dsRNA construct was confirmed by incubation with RNAse A (B) and RNAse III (C), which specifically digest ssRNA and dsRNA, respectively. Arrows indicate the expected size products of β-actin, EF1α, Luciferase and empty L4440 which are 500 bp, 700 bp, 600 bp and 220 bp, in that order. Approximately 500 ng of dsRNA was loaded in each lane of a 1 x TBE 1.5% agarose gel. (M) 2-Log DNA marker (0.1-10 kb).

The dsRNA integrity was also verified by incubation with RNAse A (Figure 4.1, lane B) and RNase III (Figure 4.1, lane C), which specifically digest ssRNA and dsRNA, respectively. The results showed that all synthesized RNAs were cleaved by RNAse III but not RNAse A, clearly suggesting that intact dsRNA were obtained by the aforementioned method.

4.3.2. dsRNA delivery by soaking and its effect on β-actin mRNA expression levels

dsRNA's targeting the amoebae β-actin (*Np*-β-actin-dsRNA), EF1-α (*Np*-EF1α-dsRNA), as well as the non-specific luciferase (luc-dsRNA) and the empty L4440 vector (L4440-dsRNA), were administrated to cultured *N. pemaquidensis* and analysed by qRT-PCR. No reduction of β-actin mRNA levels was observed when amoebae were incubated with 2 µg/mL of *Np*-β-actin-dsRNA, at any sampling period

(Figure 4.2A, B). However, a slight but non-significant decrease in β-actin relative copy number was detected at 12 h after the 20 µg/mL dosage was administered (Figure 4.2 C, D). This reduction was significant at 24 h ($p < 0.001$), reaching a relative knockdown of approximately 84 ±7% in *Np*-β-actin-dsRNA treated samples. The silencing effect remained significant at 48 h (77 ±4%) and 72 h (88 ±7.5%).

The significant knockdown described above was not increased when a higher dosage (50 µg/mL) was used (Figure 4.2E, F). Although the β-actin relative copy number declined slightly at 24 h, the relative knockdown only reached significant levels at 48 h ($p < 0.05$), 24 h later than when the 20 µg/mL dosage was employed (Figure 4.2E, F). The most effective knockdown in samples treated with 50 µg/mL of *Np*-β-dsRNA (77 ±10.2%) was detected at 72 h ($p < 0.001$) (Figure 4.2 E), which was not significantly higher than the levels obtained, at the same sampling point, for the 20 µg/mL treatment. No major variations on β-actin expression levels were observed when either the internal (*Np*- EF1α-dsRNA) or the non-specific (luc-dsRNA) controls were added into the amoebae culture at any of the test concentrations (Figure 4.2A-F).

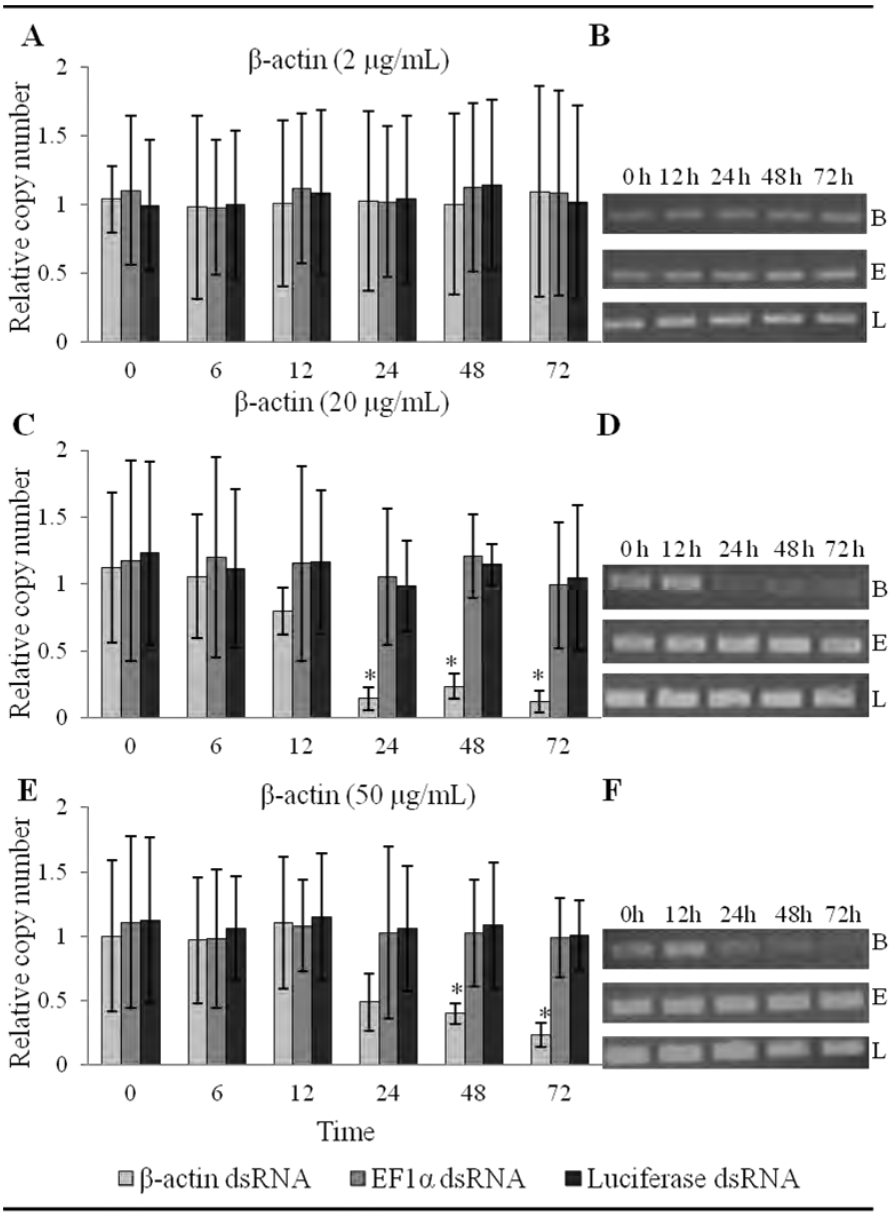

Figure 4.2 Silencing of *N. pemaquidensis* β-actin gene expression. (A), (C) and (E) qRT-PCR quantification of amoebae soaked with dsRNA at 2, 20 and 50 μg/mL, respectively. Bars symbolize the mean value ±S.E. (n= 4) at each sampling period and "*" indicate significant difference between treatments. (B), (D) and (F) RT-PCR products of β-actin mRNA in amoeba treated with *Np*-β-actin-dsRNA (lane B), *Np*-EF1α-dsRNA (lane E) and luc-dsRNA (lane C).

4.3.3. dsRNA delivery by soaking and its effect on EF1α mRNA expression levels

The same experiment described in 3.2 was performed using *N. pemaquidensis* EF1α as a target gene. In this case, *Np*-β-actin-dsRNA was used as the internal control. Similar to what was found when attempting to knockdown β-actin mRNA levels, EF1α silencing was not triggered when the lower concentration of *Np*-EF1α-dsRNA (2 µg/mL) was administered (Figure 4.3A, B). The EF1α expression levels also remained significantly stable throughout the experiment when the 20 µg/mL dosage was employed (Figure 4.3C, D). However, significant relative knockdown of EF1α was achieved at 48 h (83 ±7.3%) post the administration of *Np*-EF1α-dsRNA at the concentration of 50 µg/mL ($p < 0.05$) (Figure 4.3E, F). This silencing effect did not remain significant for longer than 24 h (Figure 4.3E).

4.3.4. Daily administration of dsRNA does not improve effectiveness of knockdown

Aiming to improve the silencing duration and efficiency observed previously, 20 µg/mL of each dsRNA was administered daily to the amoeba culture for a period of seven days. The experiments were carried out under the same conditions as detailed before with sampling performed at 0, 24, 48, 72 h and 7 days after the first dsRNA addition. The daily administration of *Np*-β-actin-dsRNA did not improve the effectiveness of β-actin downregulation, when compared to a single administration of 20 µg/mL (Figure 4.4A, B).

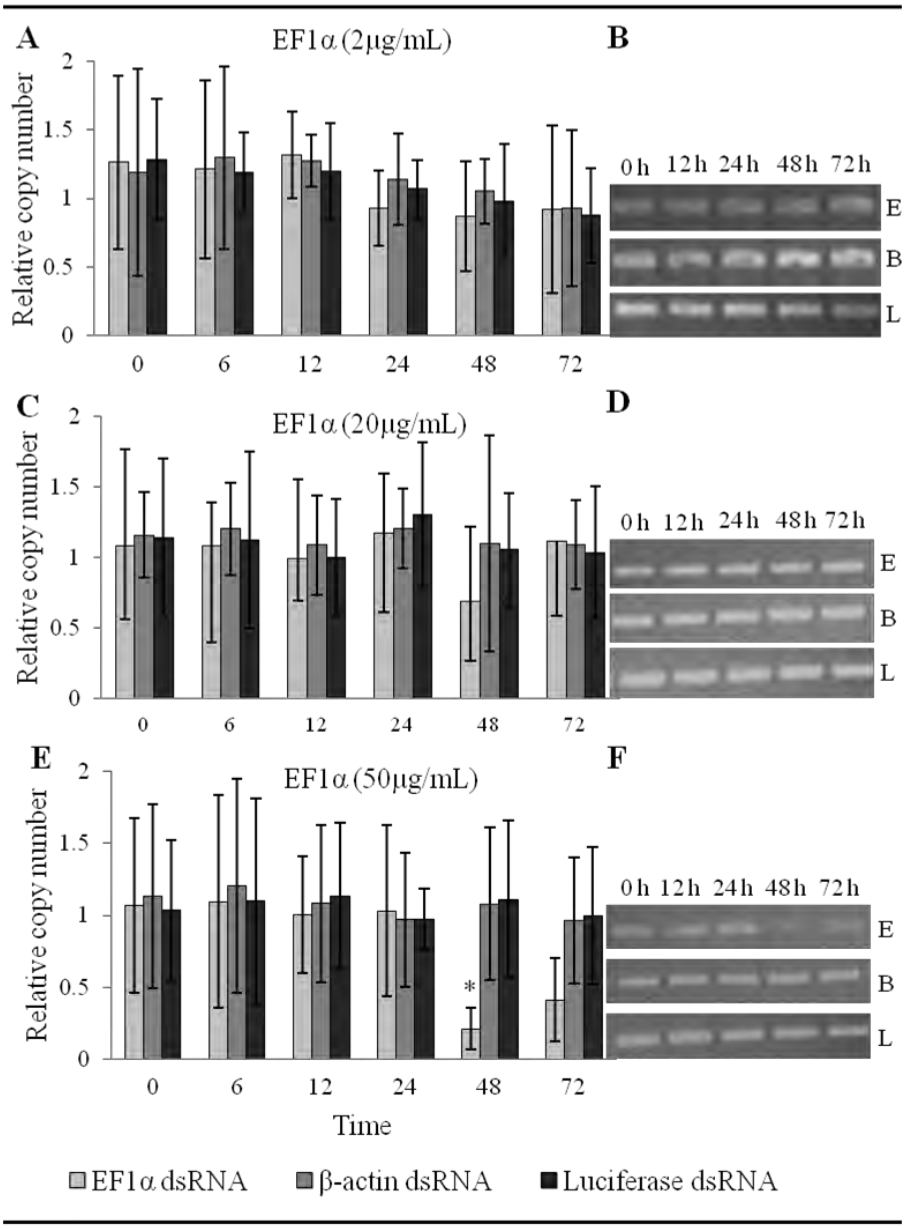

Figure 4.3 Silencing of *N. pemaquidensis* EF1α gene expression. (A), (C) and (E) qRT-PCR quantification of amoebae soaked with dsRNA at 2, 20 and 50 μg/mL, respectively. Bars symbolize the mean value ±S.E. (n= 4) at each sampling period and "*" indicate significant difference between treatments. (B), (D) and (F) RT-PCR products of EF1α mRNA in amoeba treated with *Np*-EF1α-dsRNA (lane E), *Np*-β-actin-dsRNA (lane B), and luc-dsRNA (lane C).

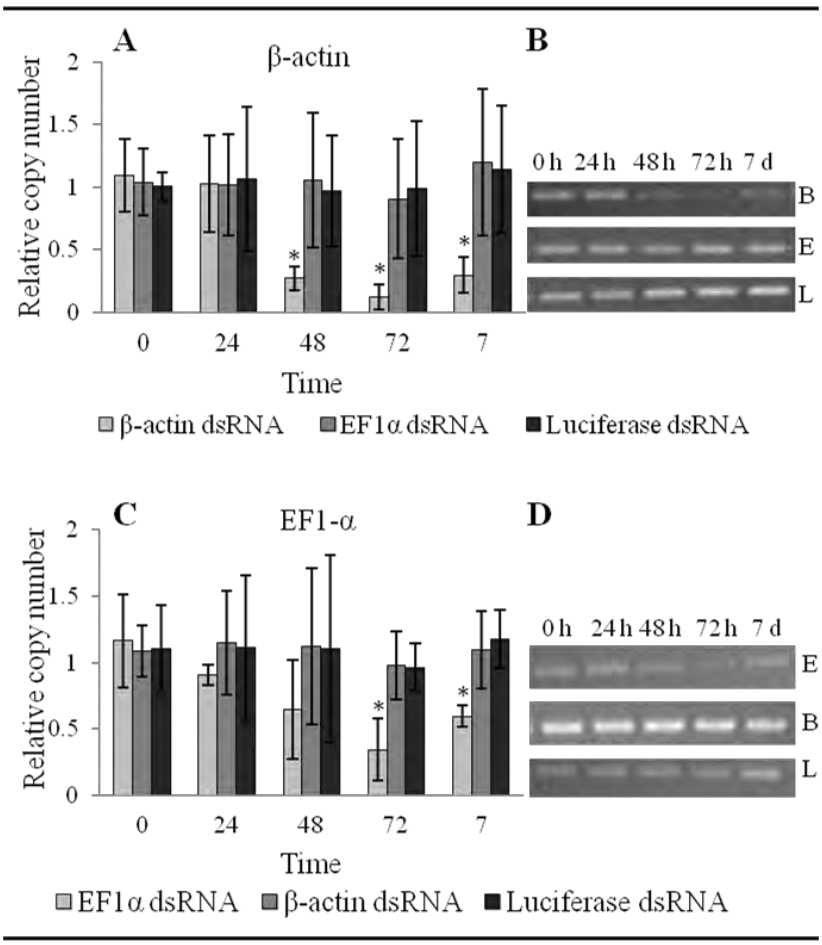

Figure 4.4 Daily administration of bacterially expressed dsRNA (20 µg/mL). (A) and (C) β-actin and EF1-α qRT-PCR quantification. Bars symbolize the mean value ±S.E. (n= 4) at each sampling period and "*" indicate significant difference compared with controls. (B) and (D) RT-PCR products of β-actin and EF1α in amoeba treated with *Np*-EF1α-dsRNA (lane E), *Np*-β-actin-dsRNA (lane B), and luc-dsRNA (lane C).

Moreover, the silencing effect triggered by the daily introduction of *Np*-β-actin-dsRNA was delayed by 24 h, with significant downregulation only observed from 48 h ($p < 0.05$) (Figure 4.4A). At 72 h, the β-actin mRNA expression reached the lowest level, with a relative knockdown of 89 ±7.63%. The observed reduction remained significant until the end of the experiment (Figure 4.4A). On the contrary, while no silencing effect was detected when amoebae received an one-off 20 µg/mL dose of *Np*-EF1α-dsRNA (Figure 4.3C), a significant downregulation of EF1α was

observed at both 48 and 72 h (70 ±11.29% and 41 ±4.37%) when the same dosage was administered daily to the amoeba culture ($p < 0.01$) (Figure 4.4C, D). However, the target gene depletion was not significantly superior to when the 50 µg/mL dosage was employed (83 ±7.3% at 48h) (Figure 4.3 3E). Similarly to previous observations, both the β-actin and EF1α mRNA levels were not affected by administration of control dsRNA's (Figure 4.4 A-C).

4.3.5. Suppression of β-actin mRNA levels induces unexpected phenotypic changes in N. pemaquidensis

Image based analysis of dsRNA-treated trophozoites showed that all β-actin depleted amoebae developed into an unexpected dormant cyst-like phase (Figure 4.5A, B), which has not been previously described in species from the genus *Neoparamoeba* [298-300]. The round shape encysted amoeba were approximately 15 µm in diameter and, when analysed under 100x magnification, small spherical structures similar to nuclei could also be observed within each individual (Figure 4.5C). Unlike trophozoites, cysts were immobile and attached to the substratum.

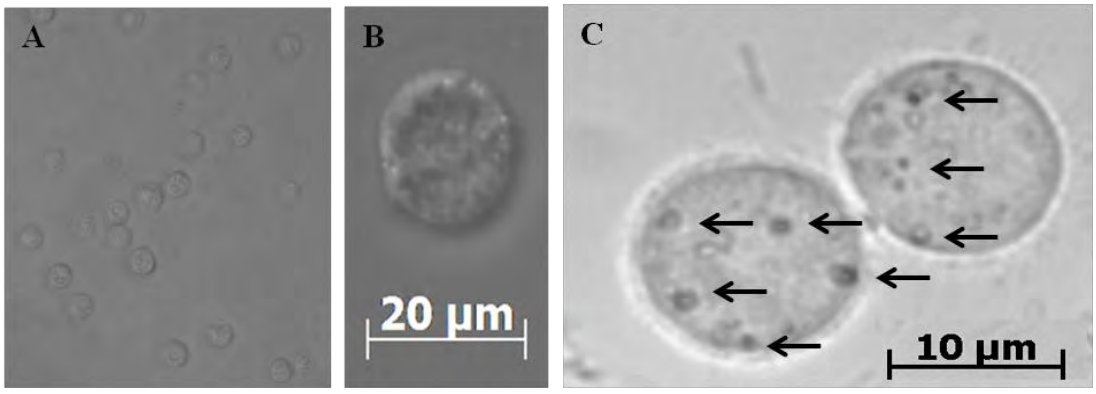

Figure 4.5 Phenotypic changes of *Np*-β-actin-dsRNA-treated amoeba. Images (A and B) were obtained under 10 and 40x objective, respectively. (C) Cyst-like amoeba under 100x magnification. Several dark spots that resemble nuclei could be observed within each cell as indicated by arrows. Scale bar 10 and 20 µm.

While 100% of *Np*-β-actin-dsRNA treated amoeba showed noticeable phenotypic change, approximately 70% of the trophozoites soaked in 50 µg/mL of *Np*-EF1α-dsRNA showed less mobility and pseudopodia radiation, assuming a corrugated globular shape (Figure 4.6A, B). All amoebae in luciferase-dsRNA solution remained in the active feeding trophozoite stage (Figure 4.6C, D).

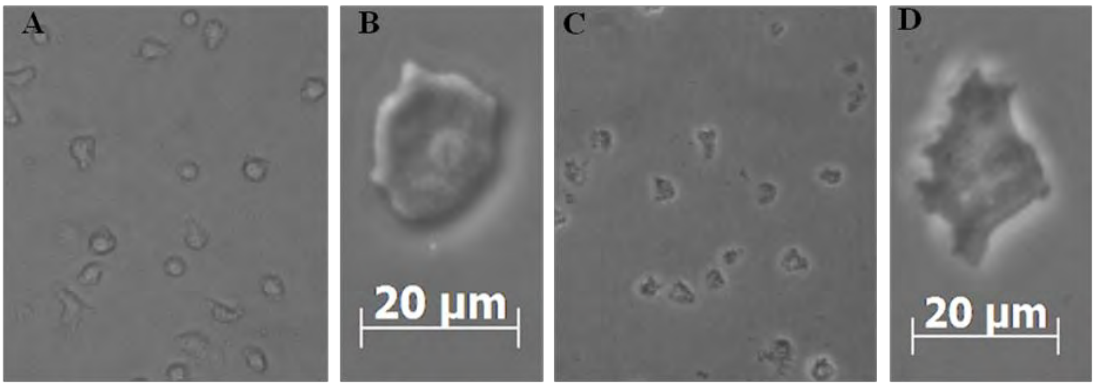

Figure 4.6 Phenotypic changes of *Np*-EF1α-dsRNA (A and B) and luc-dsRNA (c AND d) treated amoeba. Images (A and C) and (B and D) were obtained under 10and 40x objective, respectively. Scale bar 20 µm.

4.4. Discussion

In the present study we aimed to investigate whether bacterially expressed dsRNA designed against *N. pemaquidensis* β-actin and EF1α genes would trigger the degradation of targeted mRNAs when delivered by soaking. The results shown in the present report demonstrate that administration of the sequence-specific β-actin construct resulted in knockdown of the corresponding mRNA in a dose-dependent manner. Even though the β-actin mRNA levels were significantly suppressed by the higher dsRNA dose (50 µg/mL), a more effective silencing effect was observed when 20 µg/mL of *Np*- β-actin-dsRNA was delivered to the amoebae culture. The lower dsRNA concentration (2 µg/mL), on the other hand, failed to achieve a

sensible reduction of the target gene. Similarly, successful knockdown of β-actin transcript levels was not observed by Dunn et al. [301] when soaking sea anemone *Aiptasia pallida* at low dsRNA concentrations (0.5 and 0.75 μg/μL). The authors also demonstrated that deleterious phenotypic effects, including animal mortality, were displayed at the highest dsRNA concentration (1.25 μg/μL). Dose-dependent inhibition of β-actin was also reported by Pfarr et al. [302] when testing serial dilutions of dsRNA (0.035 - 35 μM) to specifically knockdown gene expression in nematode *Litomosoides sigmodontis*, by soaking. While all concentrations tested significantly inhibited β-actin expression levels, the 3.5 μM treatment induced a more consistent and less variable downregulation of the target gene. Therefore, similarly to other studies we observed a dose-dependent mRNA inhibition with dsRNA administration.

While β-actin mRNA levels were significantly suppressed by the 20 and 50 μg/mL dosages (*Np*- β-actin-dsRNA), significant downregulation of EF1α was only observed when 50 μg/mL was employed (*Np*-EF1α-dsRNA). Similarly, a significant suppression of *Entamoeba hystolica* KERP1 gene was only obtained when amoebae were soaked in 50 μg/mL of bacterially expressed dsRNA, as opposed to when the lower dosages (5 and 25 μg/mL) were administered [61]. Efficient gene inhibition by higher dsRNA dosages was also verified by Krautz-Peterson et al. [303] when soaking the parasitic platyhelminth *Schistosoma mansoni* with 10-fold serial dilutions of *in vitro* transcribed dsRNA encoding the cathepsin B gene. The fact that different target genes can respond distinctly to dsRNA dosages is widely reported in the literature. For example, different optimal dsRNA concentrations were obtained by Dunn et al. [301] when validating the administration of dsRNA constructs targeting *A. pallida* β-actin and caspase genes. A similar pattern was demonstrated in a study performed by Sukno et al. [304] where different dsRNA concentrations were

employed to assess gene expression inhibition of two transcripts from the nematode parasite *Heterodera glycines*. Although *Hg-pel-1*was most effectively suppressed at the highest dsRNA concentration used (5 mg/mL), *Hg-4E02* mRNA levels were significantly reduced only when 2.5 mg/mL was provided. Thus, it appears as though knockdown efficiency is gene specific. However, we are unsure as to why this occurs in *N. pemaquidensis* but is presumably due to either the gene itself or the efficient processing of the specific dsRNA by the RNAi machinery.

The effectiveness of β-actin downregulation was not improved by daily addition of *Np*- β-actin-dsRNA, as opposed to a single dose administration. Conversely, while EF1α mRNA levels were not influenced by the administration of a one-off concentration of 20 μg/mL of *Np*-EF1α-dsRNA, a significant downregulation of the corresponding gene was detected when the same dosage was daily incorporated to the supernatant. However, the observed inhibition was not significantly different than when 50 μg/mL of the same dsRNA construct was delivered singly. Similar results were obtained by Orii et al. [305] when soaking the planarian *Dugesia japonica* with dsRNA expressing the intermediate filament b gene (*Dj*IFb). According to the results, the successful silencing effect achieved at a high concentration of dsRNA (0.5 μg/μL) could be reproduced by repeating the treatment at a lower concentration (0.25 μg/μL) for a period of three days. Araujo et al.[306] , on the other hand, verified that triatomine bug *Rhodnius prolixus* injected with dsRNA targeting the salivary gland nitrophorin 2 (NP2) had the target gene expression level reduced by 38±7% after the first injection and by 75±14% after the second one, which was performed 48 h later.

RNAi efficiency is also known to be highly influenced by the selected target gene and its expression profile [307]. In our study we demonstrated that bacterially expressed dsRNA achieved higher RNAi efficiency when targeting β-actin,

regardless of the dosage or number of treatments employed. Similarly, Krautz-Peterson et al. [308] demonstrated that the *S. mansoni* glucose transporter 4 (SGTP4) is consistently better suppressed than SGTP1 when parasites are soaked with either siRNAs or long dsRNA in two different life stages. This may reflect differences in the ability of dsRNAs to enter internal tissues or to the differential expression of RNAi pathway components in different organs. Therefore, RNAi may not be an effective way to study all genes within a non-model organism and that the inability to effectively silence some genes may be as a result of their requirement for essential processes, somewhat acting as a protective mechanism.

Finally, it appears as though the choice of target gene influenced not only the level of knockdown but also the silencing effect persistence. While β-actin mRNA levels remained significantly lower until the last sampling period, regardless of the dosage (20 or 50 µg/mL) or number of treatments received, EF1α inhibition was detected for no longer than 24 h when the 50 µg/mL dose was delivered. However, the observed knockdown persisted for at least 4 days when we increased the administration frequency. Hussein et al. [309] demonstrated that utilisation of a smaller dsRNA corresponding to secreted acetylcholinesterase B (AChE B) from the parasitic nematode *Nippostrongylus brasiliensis* resulted in a more powerful and persistent suppression of AChE B secretion than when animals were soaked with a dsRNA construct designed against the full length encoding AChE B. According to the authors, the results suggest that smaller dsRNAs are more efficiently incorporated by these parasites. This might help explain why *Np*- β-actin-dsRNA (500 bp) was able to trigger a longer and more effective silencing effect than *Np*-EF1α-dsRNA (700 bp).

Further microscopy analysis demonstrated that administration of dsRNA targeting the candidate genes distinctly affected cell morphology of treated amoeba.

Most importantly, introduction of *Np*-β-actin-dsRNA led to development of dormant spherical cells that resemble cysts. Encystation is one of the survival strategies employed by some species of amoeba, in which, under unfavourable external conditions, trophozoites lose their characteristic pseudopoidal movement and get endowed with a protective and impenetrable wall [310]. Cysts are immotile and highly resistant, remaining metabolically inert as long as conditions are unfavourable for hatching [311]. Other amoeba species, on the other hand, do not form cysts but can rapidly assume a non-motile rounded shape, which survives for shorter periods of time without any evidence of activity. This stage is called a pseudocyst and differs from the true cyst by the absence of any cyst wall and by the consequent reduction in survival capacity [312].

Development of pseudocyst as a response to starvation and chemical exposure has been reported in the amoebae *Hydramoeba hydroxena* [313] and *Acanthamoeba* spp. [314, 315], respectively. According to those authors, despite the lack of cell wall, the pseudocysts morphologically resembled immature cysts, which prevent them to be identified by visual analysis alone. Therefore, as formation of neither cyst nor pseudocyst has been previously reported in species from the genus *Neoparamoeba*, supplementary studies on the morphology and histology of the observed cells need to be undertaken to validate if *N. pemaquidensis* is capable of forming such structures.

Despite not being as drastic as in *Np*-β-actin-dsRNA treated amoeba, morphological changes were also evident within the *Np*-EF1α-dsRNA group. However, only a percentage of the amoebae showed the observed phenotype, which may explain the fact that no significant knockdown was detected at 72 h by the qRT-PCR analysis, while a fainter band was visualized when the PCR product of samples soaked in 50 μg/mL of *Np*-EF1α-dsRNA was analysed on an agarose gel. No

reasonable explanation was found to justify this inconsistency, as the dsRNA solution was evenly diluted in the media. Considering the administration of dsRNA targeting luciferase was not able to elicit any visible phenotypic changes in *N. pemaquidensis*, we believe that the morphological responses described above were associated with the target genes' suppression and not to a toxic effect caused by the dsRNA exposure.

In conclusion, the present work provides strong evidence that dsRNA administrated via immersion was able to successfully reduce *N. pemaquidensis* β-actin and EF1α mRNA levels, showing significant changes in phenotype. The inhibition extent and persistence was both gene and dose-dependent and increased dsRNA administration did not necessarily improve downregulation efficacy for β-actin. The silencing effect demonstrated in our study was also gene-specific, as amoebae treated with both external controls (luciferase-dsRNA and L4440-dsRNA) showed no significant changes in expression of either β-actin or EF1α mRNA levels. Additionally, dsRNA targeting β-actin had no effect on EF1α expression levels and *vice versa*. Unfortunately, we were not able to validate the knockdown effect at the protein level, as there are currently no *N. pemaquidensis* antibodies to either β-actin or EF1α available at the present time. Although preliminary, this is the first evidence for the presence of an active RNAi machinery in at least one member of the *Neoparamoeba* genus. In the future, we plan to replicate the experiments using *N. perurans* and, therefore, investigate the possibility of RNAi machinery retention or loss within the genus. Additionally, the identification of RNAi related genes using comparative genomic studies will be imperative to confirm and better comprehend the RNAi pathway in *Neoparamoeba* species (Chapter 3 and 6). This is particularly important as RNAi-mediated technology could assist not only to identify and

characterize new targets for AGD intervention, but also to uncover novel aspects of amoeba biology, mechanisms of infection and host response.

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CHAPTER 5: Experiment – 3

Ingestion of bacterially expressed dsRNA induces target gene-dependent knockdown in species from the genus *Neoparamoeba*

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Submitted to: Aquaculture

Abstract

RNA interference (RNAi) mediated by double stranded RNA (dsRNA) has emerged as one of the most promising techniques to study gene function of non-model protozoan parasites. In Chapter 4, we have previously demonstrated that bacterially expressed dsRNA delivery by immersion elicited successful knockdown in *Neoparamoeba pemaquidensis*, the non infective strain of the causative agent of salmonid amoebic gill disease (AGD). However, considering that amoeba naturally feeds on microorganisms, direct ingestion of bacteria engineered to express dsRNA would allow rapid and low-cost analysis of gene function on large-scale. Therefore, the main objective of the present study was to investigate if oral administration of bacteria expressing dsRNAs would also induce suppression of *N. pemaquidensis* β-actin and EF1α. Unexpectedly, no significant variation of EF1α relative copy number was triggered by dsRNA ingestion, despite effective bacterial uptake. β-actin, on the other hand, presented similar silencing efficiency than when the same construct was delivered by soaking. However, the observed RNAi response was delayed by at least

72 hours. Surprisingly, no phenotypic changes were verified in β-actin-depleted amoeba. The results all together show that oral delivery of bacterially expressed dsRNA can successfully induce RNAi responses in *N. pemaquidensis*, albeit not as efficient as by soaking. Therefore, further investigation is required to develop more efficient and specific RNAi delivery systems in *Neoparamoeba* species.

Keywords: aquaculture; amoebic gill disease; salmon; RNA interference; feeding

5.1. Introduction

Double stranded RNA (dsRNA) - mediated interference (RNAi) has emerged as a powerful tool for rapid analysis of gene function [85], especially in organisms that are not amenable to classical genetic approaches [283]. This technique allows investigators to suppress specific genes of interest using RNAi triggers to mediate target-specific mRNA destruction resulting in transcriptional suppression [84]. RNAi can be elicited either when exogenous long dsRNAs are introduced into the cell or when endogenous dsRNAs or short hairpin RNAs (shRNAs) are produced intracellularly [316]. Once inside the cell, the RNAi precursors are then processed into 21-25 bp small-interfering RNAs (siRNAs) by an RNaseIII endoribonuclease enzyme called Dicer [317]. The short RNA duplexes will subsequently unwind and become incorporated into a large Argonaute (Ago) containing effector complex, called RNA-induced silencing complex (RISC), which guides the degradation of complimentary mRNA leading to translational repression of the target gene [92].

RNAi has been successfully used to investigate gene function in a variety of organisms ranging from unicellular protozoans, insects and fungi to complex organisms such as plants and vertebrates [56, 87, 318-320]. Numerous methods have been developed to facilitate delivery of RNAi triggers into organisms, each with their own set of advantages and disadvantages [321]. Direct microinjection is the most commonly used procedures for delivery of dsRNA [322]. However, the invasive nature of such technique often results in direct mortality, especially when large volumes are employed [307]. Therefore, the development of a non-disruptive technique that preserves the integrity of the treated animal is highly desired. Soaking the target organism in solution containing *in vitro* synthesised dsRNA is a non-invasive and effective strategy to deliver RNAi [323]. However, this technique is not

considered feasible for large-scale purposes, as high concentrations of dsRNA are necessary to ensure continuous suppression of the target gene [264]. In this context, RNAi by feeding facilitates large-scale RNAi screening as it is less labour-intensive and less expensive than the other methods [324].

Successful gene downregulation by oral administration of bacteria expressing dsRNA was first reported in *Caenorhabditis elegans* by Timmons and Fire [325]. Construction of bacterial RNAi feeding construct implies cloning a gene-specific DNA fragment into a plasmid vector (L4440) containing two T7 promoters in opposite orientation, followed by transformation into bacterial strain HT115(DE3), an RNase III-deficient strain of *E. coli* with IPTG inducible expression of T7 polymerase [326]. Since first described, this bacteria-mediated delivery of dsRNA has been successfully reported in planarians [327], amoeba [61], paramecium [328], marine and freshwater sponges [329], insects [322, 330] and shrimp [49].

The protozoan parasite *Neoparamoeba perurans* is the causative agent of amoebic gill disease (AGD) in marine farmed Atlantic salmon [13]. The disease is the major health concern affecting the salmonid aquaculture industry in Tasmania, Australia [8]. At the moment, the only effective treatment available is freshwater bathing [23], which contributes to higher production costs up to 20% due to its effect upon fish performance and the additional labour and freshwater requirements [27]. As a result, the development of improved therapeutical strategies for coping with this disease is imperative for the continued sustainability of the Tasmanian Atlantic salmon aquaculture industry. Therefore, the investigation of *N. perurans* gene function through RNAi technology would contribute to improve our understanding of the amoeba biology and mechanisms of infection, which is crucial for the development of novel intervention strategies against AGD.

In the previous chapter, we demonstrated for the first time that administration of purified bacterially expressed dsRNA by soaking successfully downregulated the mRNA expression levels in *Neoparamoeba pemaquidensis*, the non infective strain closely related to the causative agent of AGD (*Neoparamoeba perurans*). However, considering the dsRNA longevity in seawater is unknown and often continuous dsRNA exposure is required to achieve long-lasting suppression of the target gene, we believe that administration of dsRNA through bacterial ingestion would provide a direct, continuous and cost-effective RNAi delivery system. This method could be particularly advantageous when studying organisms that naturally feed on bacteria, such as amoebae. Therefore, the aim of the present study was to investigate whether ingestion of bacteria expressing dsRNA would improve suppression of gene expression in species from the genus *Neoparamoeba*. Similar to previous studies, the experiments were performed using *N. pemaquidensis*, as the *in vitro* culture of *N. perurans* only became available recently [36].

5.2. Material and Methods

5.2.1. *Neoparamoeba pemaquidensis* culture conditions

N. pemaquidensis originally isolated and cloned from AGD affected salmon were used in the present experiment. The amoeba were grown on malt-yeast-seawater agar plates (75% filtered seawater, 25% distilled water, 0.01% malt , 0.01% yeast, 2% bacto agar) and kept at 16°C, as previously described [331]. Prior to each feeding experiment, the monolayer of amoeba was removed from agar plates using a transfer pipette and sterile seawater, followed by three low-speed centrifugation steps (400 x g, 2 min) to eliminate bacterial load naturally present in agar culture. The

supernatant containing the amoeba was submitted to a final centrifugation step at 4000 x g for 5 min and amoeba pellet resuspended in sterile seawater. Once enumerated by haemocytometer count, the amoeba trophozoites were evenly spread into 24 wells culture plates (Nunclon™ Delta Surface) to a final concentration of 10^5amoebae/mL/well. Plates were incubated at 16°C until the end of each experiment.

5.2.2. RNA isolation and reverse transcription

Total RNA was isolated from *N. pemaquidensis* cells using Trizol® (Invitrogen), according to the manufacturer's instructions. RNA was quantified using a NanoDrop ND-1000 Spectrophotometer and its integrity assessed on 1.5% TAE agarose gel. Residual contaminating DNA was digested using TURBO DNA-free™ (Ambion) and first-strand cDNA synthesized using Superscript™ III Reverse Transcriptase with oligo(dT) primer (Invitrogen).

5.2.3. Synthesis of bacterially expressed dsRNA

As we were aiming to investigate whether the administration of bacterially expressed dsRNA by feeding would elicit a more effective silencing effect than by soaking, the same target genes (β-actin and EF1α) and external controls (luciferase and empty L4440 vector), as well as specific primer pairs, used in soaking experiments (Chapter 4, section 4.2.5) were employed in the present study. Briefly, β-actin, EF1α and luciferase fragments that were amplified using Phusion DNA polymerase (Invitrogen) and cloned into pAcquire vector (Gene Works) were digested with Nhe I and Spe I (New England BioLabs) and subcloned into the double

T7 promoter vector PL4440. Following cloning, the nucleotide sequences of the recombinant plasmids were confirmed by DNA sequencing and the resulting constructs transformed into HT115 (DE3) RNase III-deficient *E. coli* strain, which is modified to express T7 RNA polymerase from an isopropyl-β-D-thiogalactopyranosine (IPTG)-inducible promoter. In addition to luciferase, an empty L4440 vector without any insert was also transformed into HT115 (DE3) and used as negative control. Individual colonies carrying the plasmids were inoculated in LB broth containing ampicillin (100 μg/mL) and tetracycline (12.5 μg/mL) and grown overnight at 37 °C, with shaking. Cultures were diluted 1:50 in 2YT medium containing the same antibiotics and allowed to grow to OD_{600} ~ 0.6. IPTG was added to 2 mM for initiation of dsRNA synthesis and cultures induced for 4 h incubation at 37 °C. The bacterial cells were harvested by centrifugation at 6000 x g for 10 min at 4 °C.

5.2.4. dsRNA validation

To confirm that dsRNA was successfully expressed in the bacteria, dsRNA corresponding to each target gene and controls were purified from the bacteria using the same method previously employed by us in soaking experiments (Chapter 4, table 4.1). The protocol briefly consisted of total RNA isolation using Trizol® (Invitrogen), followed by Turbo DNase and RNase A (Ambion) incubation to remove contaminating genomic DNA and single-stranded RNA, respectively. Double-stranded RNAs were analysed by agarose gel electrophoresis and concentration determined using a NanoDrop ND-1000 Spectrophotometer. The dsRNA integrity was further confirmed by ShortCut RNAse III (New England Biolabs) digestion.

5.2.5. Delivery of dsRNA-expressing bacteria via feeding

Prior the experiments, the amoebae were transferred to 24-well culture plates (Nunclon^{TM} Delta Surface) containing 1 mL of filtered seawater in each well. The treatments were performed in quadruplicate and each replicate comprised all the amoebae within a single well (10^5 amoebae). Bacterial inoculum expressing *N. pemaquidensis* β-actin and EF1α, as well as the unrelated luciferase and the empty L4440 vector were added to respective wells to obtain a final ratio of 10^4 bacteria per amoeba. The selected amoeba/bacteria ratio was based on a study performed by Solis et al. [61] when using similar methodology to silence gene expression in *Entamoeba histolytica.* Cellular density of bacterial inoculums was determined assuming that an optical density of 1 at 600 nm corresponds to 10^8 bacteria/mL [61]. Amoebae fed once with dsRNA-expressing bacteria were cultured under standard *in vitro* conditions and sampled at 0, 24, 48, 72 h and 7 days post introduction of bacteria producing dsRNA.

5.2.6. Total RNA extraction and RT-PCR

At each sampling period, the seawater from the sampled wells was removed by pipetting and each well rinsed twice with filtered seawater to eliminate unattached debris and non-ingested bacteria. The amoebae were then detached from the culture plates by adding Trizol® (Invitrogen) and transferred to fresh 1.5 mL tubes. Total RNA was isolated from dsRNA treated amoebae as described previously, followed by Turbo DNAse-treatment (Ambion) and reverse transcription using Superscript III (Invitrogen). To determine whether β-actin and EF1α transcripts were effectively silenced, reverse transcription PCR (RT-PCR) was conducted using the synthesized

cDNA as a template in a reaction containing GoTaq® (Promega) and the target genes' specific primers. The temperature profile for PCR amplification was performed by holding at 94 °C for 5 min, followed by 28 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min; and a final extension at 72 °C for 7 min. The PCR products were analysed by agarose gel electrophoresis and the mRNA expression of the target genes evaluated using the intensity of the bands from luciferase-dsRNA treated samples as a control.

5.2.7. Quantitative real time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed on an ABI 7600 system to determine whether the administration of bacterially expressed dsRNA targeting the *N. pemaquidensis* β-actin and EF1α would significantly downregulate the mRNA expression levels of the target genes. Amplification of both target genes was obtained by the same qRT-PCR primers pairs used in the soaking experiments (Chapter 4, table 4.1). All reactions were performed in triplicate, each containing 4 µL of diluted cDNA, 2 x SensiMix SYBR (Bioline) and 0.5 mM forward and reverse primers in a 10 µL reaction. The amplification profile consisted of an initial denaturation step at 95 °C for 10 min; 40 cycles of 95 °C for 20 s, 58 °C for 20 s and 72 °C for 20 s, followed by a dissociation stage according to the manufacturer's instructions. Absolute quantification of β-actin and EF1α was determined by generating external standard curves using a 10-fold serial dilution of plasmid DNA as templates.

The absolute amount of each target gene was expressed as copy number using the following equation:

$$\textbf{Quantity} = 10^{(Ct-b)/m}$$

Where Ct is the threshold cycle, b is the y-intercept and m is the slope of the linear regression equation obtained from each standard curve. The efficiency of each primer set was calculated from the formula $E = 10^{-1/slope}$. The expression levels of β-actin and EF1α were presented as relative copy number which was normalized against the samples that received empty L4440 vector treatment.

5.2.8. Statistical analysis

Significant variation in β-actin or EF1α expression levels within treatments, at each sampling period, was calculated by one-way analysis of variance (ANOVA) using the R v.2.14 software (R Development Core Team 2007). Values were considered to be significant at $p < 0.05$. All numerical data are expressed as the mean ± standard error.

5.2.9. Validation of dsRNA expressing bacteria ingestion

In order to differentiate the administered bacteria from those naturally present in the amoeba culture, the *E. coli* expressing dsRNA were stained using propidium iodide (PI), a nucleic acid binding fluorescent dye. Fluorochrome stock solution of PI was prepared in phosphate buffered saline (PBS) at 500 µg/mL and filtered with 0.2 µm Millipore filter. Approximately 1 mL of IPTG induced bacteria was harvested by centrifugation (6000 x g, 3 min), followed by overnight formaldehyde fixation at 4 º C. The bacterial pellet was incubated with PI (3µg/mL) for 30 min in the dark and

washed several times with PBS to remove unbound dye. Successful bacterial PI fluorescence was verified using a Zeiss Axio Observer inverted microscope under Rhodamine filter set (excitation 540–552 nm, emission 575–640 nm) and 100x oil-immersion objective. Stained bacteria and trophozoites were associated for two hours under standard culture conditions, at the same ratio described at section 5.2.5. Following the incubation period, amoebae and bacteria were separated by low-speed centrifugation (400 x g, 2 min) and the fraction containing the trophozoites resuspended in PBS. Using the same microscope as above, trophozoites and stained bacteria within food vacuoles were identified by phase contrast (40x, Ph 2) and fluorescence (40x, Rhodamine filter set), respectively. Images were acquired with a Zeiss Axiocam CCD camera and Zeiss AxioVision 4.8.1 software (Zeiss Germany).

5.3. Results

5.3.1. Verification of dsRNA integrity

Successful production of dsRNA in *E. coli* HT115 was validated by analysing purified dsRNAs on 1% agarose gel before and after RNAse A and RNAse III digestion. As shown in Figure 5.1, all synthesised dsRNAs were resistant to RNAse A digestion, but susceptible to RNAse III, indicating that good quality dsRNA was obtained for this study. Prominent bands of about the expected size were observed for dsRNA constructs targeting *N. pemaquidensis* specific genes and non-specific luciferase, while a small product of approximately 220 bp was detected in samples extracted from bacteria transformed with empty L4440 vector (Figure 5.1).

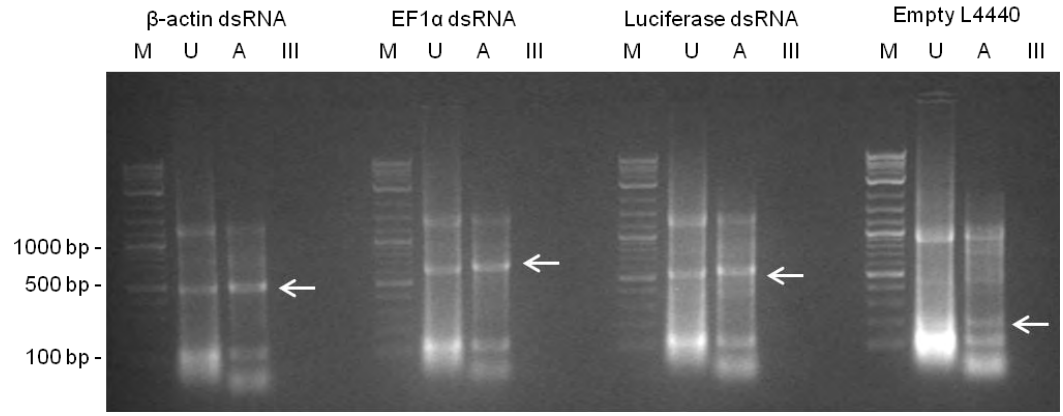

Figure 5.1 Agarose gel electrophoresis of dsRNA purified from the *E. coli* HT115(DE3). Following TRIZOL® extraction, dsRNA integrity was assessed by submitting untreated samples (U) to RNAse A (A) and RNAse III (III) digestion. As expected, dsRNA was resistant to RNAse A and susceptible to RNAse III, confirming that good quality dsRNA was produced in the bacteria. Arrows indicate the expected size products of β-actin (500 bp) EF1α (700 bp), Luciferase (600 bp) and empty L4440 vector (220 bp). (M) 2-Log DNA marker (0.1-10 kb).

5.3.2. Target gene downregulation by ingestion of bacterial dsRNA

E. coli strain HT115 genetically engineered to express dsRNA targeting *N. pemaquidensis* β-actin and EF1α dsRNA, as well as luciferase and empty L4440 vector were directly fed to the amoeba trophozoites to a final concentration of 10^4 bacteria/amoeba. To confirm that ingestion of dsRNA triggered specific RNAi in *N. pemaquidensis*, qRT-PCR was performed to detect the mRNA level of both target genes at each sampling period. When β-actin knockdown was assessed, samples treated with EF1α-dsRNA were regard as internal controls and *vice versa*.

Quantitative analysis of gene expression demonstrated that significant knockdown of β-actin mRNA transcripts was only detected one week after the bacteria expressing β-actin dsRNA was introduced to the amoeba culture (Figure 5.2A). At 7 days, the target gene mRNA levels was reduced by 83 ±6.63% as

opposed to samples submitted to either internal (EF1α-dsRNA) or external (luciferase-dsRNA) controls.

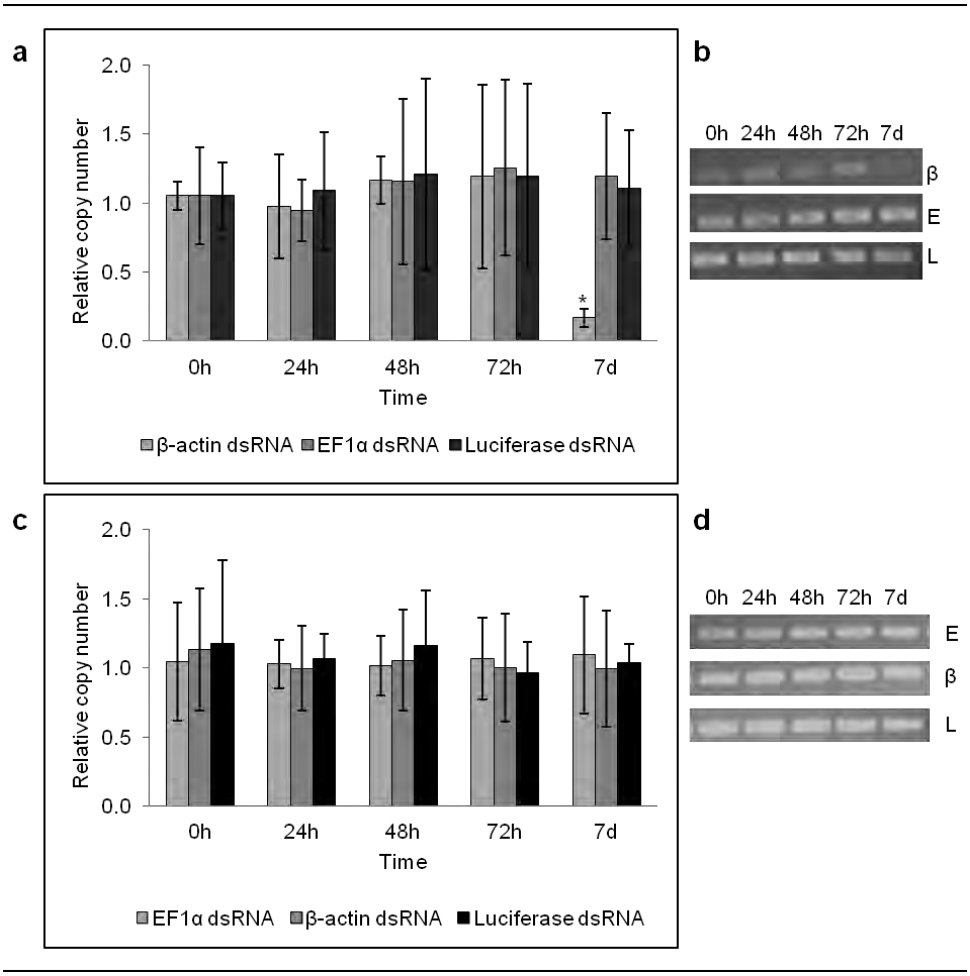

Figure 5.2 Gene knockdown in *N. pemaquidensis* orally administrated with bacteria expressing dsRNA designed against β-actin and EF1α. (**a**) and (**c**) represent qRT-PCR quantification of β-actin and EF1α relative copy number normalised against corresponding mRNA level of amoebae fed with empty L4440 transformed bacteria. Bars symbolize the mean value ±S.E. (n= 4) at each sampling period and asterisk "*" indicates significant difference compared to controls. (**b**) and (**d**) are representative gels of RT-PCR products of β-actin and EF1α gene expression at each sampling period. Row E – amoebae treated with *Np*-EF1α-dsRNA; Row β – amoebae treated with *Np*-β-actin-dsRNA; Row L - amoebae treated with luc-dsRNA.

The observed suppression was supported by RT-PCR analysis, where a less intense band was detected in β-actin-dsRNA treated group, at the same sampling period (Figure 5.2B). Conversely, EF1α expression levels remained significantly

stable during the entire experimental period, regardless of the treatment administered (Figure 5.2C, D). After feeding on bacteria expressing dsRNA for 7 days, no phenotypic changes were observed in trophozoites across the treatment groups (results not shown).

5.3.3. Confirmation of bacteria ingestion

To confirm that bacterially expressed dsRNA was successfully introduced into the amoeba, *E. coli* HT115 was stained with fluorescent dye and its ingestion assessed by fluorescent microscopy (Figure 5.3).

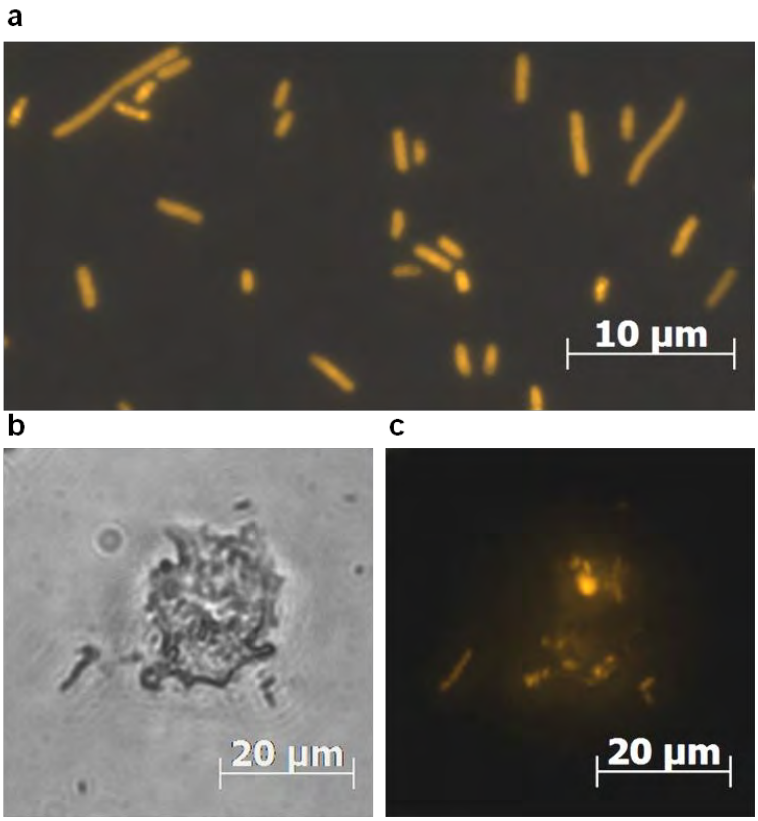

Figure 5.3 Validation of bacterially expressed dsRNA ingestion by fluorescent microscopy. (**a**) *E. coli* HT115(DE3) cell successfully stained by PI under 100x oil immersion objective (scale bar, 10 µm). Phase contrast (**b**) and fluorescent (**c**) image of the same trophozoite following 2 h incubation with stained bacteria (40x objective, scale bar, 20 µm).

Bacteria incubated for 30 min in solution containing PI at 3µg/mL appeared bright orange under the microscope, with strong fluorescent signal and low background (Figure 5.3A). Following two hours incubation, ingestion of stained bacteria was successfully confirmed under fluorescence excitation filter. Trophozoites showed fluorescent food vacuoles, as well as intact rod-shape bacteria in the cytoplasm (Figure 5.3B, C). A light orange background could also be observed in some areas of the trophozoites, which appears to be soluble products of bacterial digestion that were diffused into the cytoplasm. Rapid uptake, and possible digestion, of dsRNA expressing bacteria was verified in the majority of trophozoites. However, a small fraction of amoeba presented no fluorescence signal, suggesting that the dsRNA was not equally taken up by all the trophozoites.

5.4. Discussion

In Chapter 4, we have previously demonstrated that bacterially expressed dsRNA administered via immersion was able to successfully reduce *N. pemaquidensis* β-actin and EF1α mRNA levels. However, given that *Neoparamoeba* naturally feeds on bacteria through phagocytosis, we investigated whether direct ingestion of dsRNA-expressing bacteria would elicit similar suppression in *N. pemaquidensis*. As we were aiming to compare the efficiency of both delivery methods, *N. pemaquidensis* β-actin and EF1α were again selected as target genes for these experiments.

β-actin mRNA levels were significantly suppressed when amoebae were treated with bacteria expressing the corresponding dsRNA. However, even though bacteria uptake was verified within the first hours post bacterial inoculation, the observed silencing effect was detected only at 7 days. Gene silencing appeared to be

highly specific since no reduction of the β-actin transcripts was observed in trophozoites fed with control bacteria carrying EF1α or luciferase dsRNAs. Significant dowregulation of actin by ingestion of bacteria expressing dsRNA was also demonstrated in freshwater (*Ephydatia muelleri*) and marine (*Tethya wilhelma*) sponges [329]. However, the verified knockdown was not as effective as the one observed in our study (83 ±6.63%). While *T. wilhelma* daily pulse-fed with *E. coli* expressing actin over 8 days presented a maximum inhibition of 49.6%, *E. muelleri* fed on bacterial dsRNA for a period of 24 h showed a modest, but significant, suppression of 25% [329]. The higher knockdown observed in the present study could be explained by the fact that the amoebae were continuously exposed to bacteria expressing dsRNA, as opposed to short feeding intervals.

In contrast to what was seen for β-actin, no significant variation of EF1α relative copy number was detected among the treatments analysed, at any sampling time point. The discrepancy in silencing efficiency observed amongst the candidate genes corroborates with our soaking studies (Chapter 4) where β-actin knockdown was repeatedly more effective than EF1α, regardless of the dsRNA concentration or administration frequency. The fact that not all target genes can be effectively inhibited by the ingestion of bacterial dsRNA has been previously reported in the literature. For example, Solis et al. [61] verified that while *Entamoeba histolytica* β-tubulin was successfully downregulated by the ingestion of dsRNA-expressing bacteria, there was no significant transcript reduction of the virulence factor KERP1 by the same delivery method. When investigating RNAi application in the nematode parasite *Trichostrongylus colubriformis*, Issa et al. [332] also demonstrated that bacterial feeding significantly inhibited the mRNA expression levels of tropomyosin, but not of ubiquitin. The results altogether suggest that EF1α may not be a suitable

RNAi target for *N. pemaquidensis* and, therefore, other candidate genes should be considered in further RNAi delivery studies in this species.

The β-actin relative knockdown achieved by oral administration of bacteria expressing the related dsRNA (83 ±6.63%) was not significantly different than when 20 μg/mL of the same construct was delivered by soaking either daily (89 ±7.63%) or singly (88 ±7.5%) (Chapter 4). However, despite the similarities regarding knockdown efficiency, an earlier silencing effect was observed when dsRNA was administered via immersion. While significant inhibition of β-actin was already obtained within 24 h when amoeba was soaked in solution containing 20 μg/mL of dsRNA (Chapter 4), no major variations in β-actin mRNA levels were detected earlier than 72 h when the construct was orally delivered. We were not able to accurately assess when the knockdown was first triggered, as sampling was not carried out between 72 h and 7 days. In addition to shorter kick-off period, soaking also appears to be more effective in a wider range of target genes, as EF1α transcripts were successfully downregulated by immersion (Chapter 4), but not by ingestion. Therefore, despite the logistical and cost advantages associated with feeding as a delivery method, our results demonstrate that soaking elicits more consistent and desirable RNAi silencing effect in *N. pemaquidensis*.

The divergence in efficiency observed among soaking and feeding can possibly be explained by the distinct dsRNA entry mechanisms involved in each delivery system. When delivered by soaking, dsRNA directly enters the cytoplasm potentially by endocytosis or by receptor/transporter-mediated RNA entry [61]. During feeding, on the other hand, the bacteria is engulfed by phagocytosis and ingested into food vacuoles. The surrounding lysosomes then secrete their enzymatic content into the cavity which becomes a digestive vacuole [333]. During digestion, the vacuole decreases in size as the water is withdrawn and its content becomes first

acidic (pH 5.6) and then alkaline (pH 7.3). When digestion is completed, the soluble food particles are readily absorbed into the cytoplasm by micropinocytosis [334]. Therefore, while dsRNA appears to be directly available to the amoeba when delivered via immersion, it has to resist degradation by digestive enzymes and pH variation before being possibly released into the cytoplasm, when administered by ingestion. Additionally, through soaking the dsRNA solution is evenly diluted in the water which suggests that all trophozoites are exposed to equal concentrations of dsRNA. The same is not valid for feeding where the bacterial intake and, consequently, the precise delivery dose cannot be estimated, preventing us from precisely assessing the amount of ingested bacteria required to trigger the RNAi machinery in *N. pemaquidensis*.

In the previous chapter, β-actin depleted amoeba developed into an unexpected dormant cyst-like phase, which has not been previously described in species from the genus *Neoparamoeba* [298-300]. Surprisingly, the same response was not observed in the current experiment. Even though β-actin was suppressed to a similar level than when *Np*- β-actin-dsRNA was delivered via immersion, no phenotypic changes were detected when the same construct was ingested by the amoebae. Actin is a highly conserved protein involved in a variety of essential cellular processes such as cell motility, structure and integrity [335]. In cells that undergo amoeboid motion using pseudopds, the actin rich cytoskeleton allows rapid morphological changes in response to signals from outside stimuli [336]. The cytoskeletal β-actin, one of six different actin isoforms identified [337], is known for occupying the cytoplasm immediately above the cell membrane [338, 339]. In amoeba, β-actin is found associated with plasma membrane as a non-membranous component [340], which might explain why phenotypic changes were observed only

during the soaking experiment, when the trophozites plasma membrane was in direct contact with the dsRNA solution.

In conclusion, our study demonstrates that bacteria expressing β-actin-targeted dsRNA are successfully ingested by *N. pemaquidensis*, resulting in effective knockdown of the corresponding mRNA. The observed silencing effect was target-dependent, as only β-actin was significantly suppressed by dsRNA ingestion. The downregulation was gene-specific as neither the external (luciferase-dsRNA and L4440-dsRNA) nor the internal (*Np*- EF1α-dsRNA) controls had significant effect on β-actin relative copy number. Unfortunately, we were not able to validate the suppression at the protein level, as there are no *N. pemaquidensis* antibodies to β-actin available at the present time. Despite being more convenient and affordable, RNAi delivery via ingestion does not appear the ideal delivery method to amoebae. Apart from triggering a delayed silencing response, it leads to an uneven dsRNA uptake which can cause a higher variability within treatments. The present study provides additional evidence for the presence of functional RNAi machinery in *N. pemaquidensis*, as well as laying the foundation for further research on the development and optimization of RNAi methodologies to study practical aspects of *Neoparamoeba* biology, such as virulence in *N. perurans*.

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CHAPTER 6: Experiment - 4

Foundation studies on RNAi-based approach in *Neoparamoeba perurans*

A viable RNAi model system for the non-infective strain of *Neoparamoeba* spp. was validated in previous chapters. Such findings have provided valuable tool for further application of RNAi technology in the aetiological agent of amoebic gill disease (AGD), especially now that a simple and effective *in vitro* culture method for *Neoparamoeba perurans* has been developed [36]. Therefore, the present chapter addresses the current status of foundation studies on RNAi-based approach in *N. perurans*.

6.1. Validation of *N. perurans in vitro* culture

Trophozoites freshly isolated from the gills of AGD-infected fish were cultured on malt-yeast-seawater plates overlaid with sterile seawater, at 16 °C, according to Crosbie et al. [36]. As previous efforts to grow *N. perurans* have shown that amoebae often loses its virulence under culture condition [13], the identity of cultured trophozoites was confirmed by PCR analysis using specific oligonucleotide primers against the *N. perurans* gene 22CO3 (f.5'-CACCCTGAGCTATTCTCGACAATTTG-3' and r.5'-TTACAACTCAGCTTTTGGAACA-3') and the *Neoparamoeba pemaquidensis* 18s rRNA gene (f.5'-CTGACTGCTTTCGGGTAG-3' and r.5'-ACTCATCACTCACCCCAACG-3'). The monolayer of amoeba was removed from the plates using the same method described in previous chapters. RNA from cultured

trophozoites was isolated using RiboPure™ kit (Ambion), followed by Turbo DNAse-treatment (Ambion) and reverse transcription (Superscript™ III Reverse Transcriptase, Invitrogen). The resulting cDNA was used as template in a PCR reaction containing GoTaq® (Promega) and the species-specific primers. The identity of freshly isolated trophozoites was confirmed by gel electrophoresis, where a specific single band was obtained when *N. perurans* primers were used in the PCR reaction (Figure 6.1). No amplification product was obtained with either *N. pemaquidensis* 18s rRNA primers and no template controls (Figure 6.1), confirming the presence of *N. perurans* in the culture.

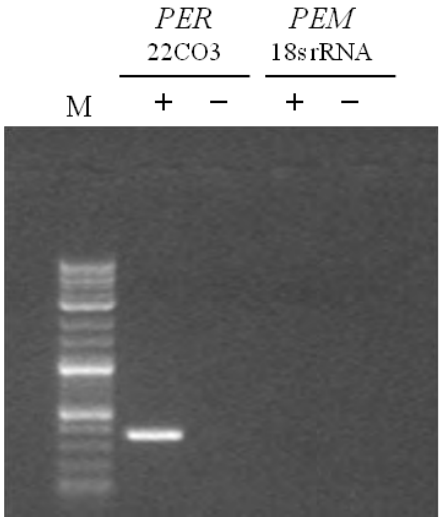

Figure 6.1 Validation of cultured trophozoites identity by PCR analysis using *N. perurans* (PER) and *N. pemaquidensis* (PEM) specific primers. (+) PCR reactions containing cDNA template; (-) no template negative controls; (M) 2-Log DNA marker (0.1-10 kb).

6.2. Identification of RNAi-associated genes in the *N. perurans* transcriptome database

Similarly to what was performed with *N. pemaquidensis* (Chapter 3), BLAST analysis of a *N. perurans* transcriptome database (M. Cook, Unpublished data) was employed to identify possible putative proteins containing conserved domains found

in members of the core RNAi machinery (see Appendix for sequences). Similarity search of annotated translated protein sequences against the UniProtKB/Swiss-Prot and NCBI non redundant protein databases revealed the absence of a typical representative Dicer candidate for *N. perurans*. Nevertheless, like in the *N. pemaquidensis* database (Chapter 3), a distinct partial amino acid (144 aa) sequence containing the Dicer DEAD-like helicase (DEXDc) domain was identified (*per*DEXDc) (Figure 6.2A). However, BLASTp alignment of both DEXDc candidates showed that these proteins are only slightly similar to one another (29% identity, 7^{e-4}) (Figure 6.3B, C). In contrast, homology search revealed that *per*DEXDc protein shares high identity level with *Arabidopsis thaliana* (Q9SP32 – 38% identity, 5^{e-19}) and *Glicine max* (XP_003520888 – 39% identity, 1^{e-17}) Dicer 1 homologues (Figure 6.2B).

Transcriptome screening also revealed two protein candidates covering both conserved motifs found in the Argonaute (Ago) superfamily: PAZ and Piwi (Figure 6.2 and 6.3). Surprisingly, *N. perurans* Ago candidate 1 (1110 aa) is highly related to the *Homo sapiens* (Q8TC59.1; 24% identity, 9^{e-28}) and *Mus musculus* (Q8CDG1.2; 24% identity, 1^{e-26}) Ago-2, which were also the best-scoring hits obtained for *Np*AGO-2 (Chapter 3). The similarity between both open reading frames (ORFs) was supported by pairwise alignment, which showed that *N. perurans* candidate 1 is highly identical (77% identical, 0.0e) to *Np*AGO-2 (Figure 6.3B, C). Furthermore, the aspartate/arginine (RD) and arginine/glycine (RGG domain)-rich regions detected towards the N-terminus of *Np*AGO-2 were also observed in the *N. perurans* candidate, albeit with less number of repeats (Figure 6.3A). Therefore, candidate 1 will be regarded as *N. perurans* Ago-like 2 (*per*AGOl-2).

The Ago candidate 2, on the other hand, possesses the PAZ and Piwi domains translated in distinct reading frames, which are slightly overlapped by 38 aa (Figure

6.4A). The coding region containing the Piwi motif is highly related to *Daphnia pulex* (EFX83175; 25% identity, 1^{e-10}) and *Xenopus silurana* (A8KBF3.1; 26% identity, 8^{e-12}) Piwi-like 2, while the reading frame encoding the PAZ domain shared 26% and 25% identity with *Dugesia japonica* (BAI6794; 1^{e-07}) and *Schmidtea mediterranea* (ACC97187.1; 2^{e-06}) Piwi-like 3. Interestingly, both aa sequences showed considerably identity homology against *Np*Piwil-2 (Chapter 3), suggesting the occurrence of frame-shift errors during assembly. Therefore the protein has been named *per*Piwil-2. Pairwise alignments and the corresponding scores between *Np*Piwil-2 and each translated protein segments of candidate 2 can be observed in Figure 6.4B, C.

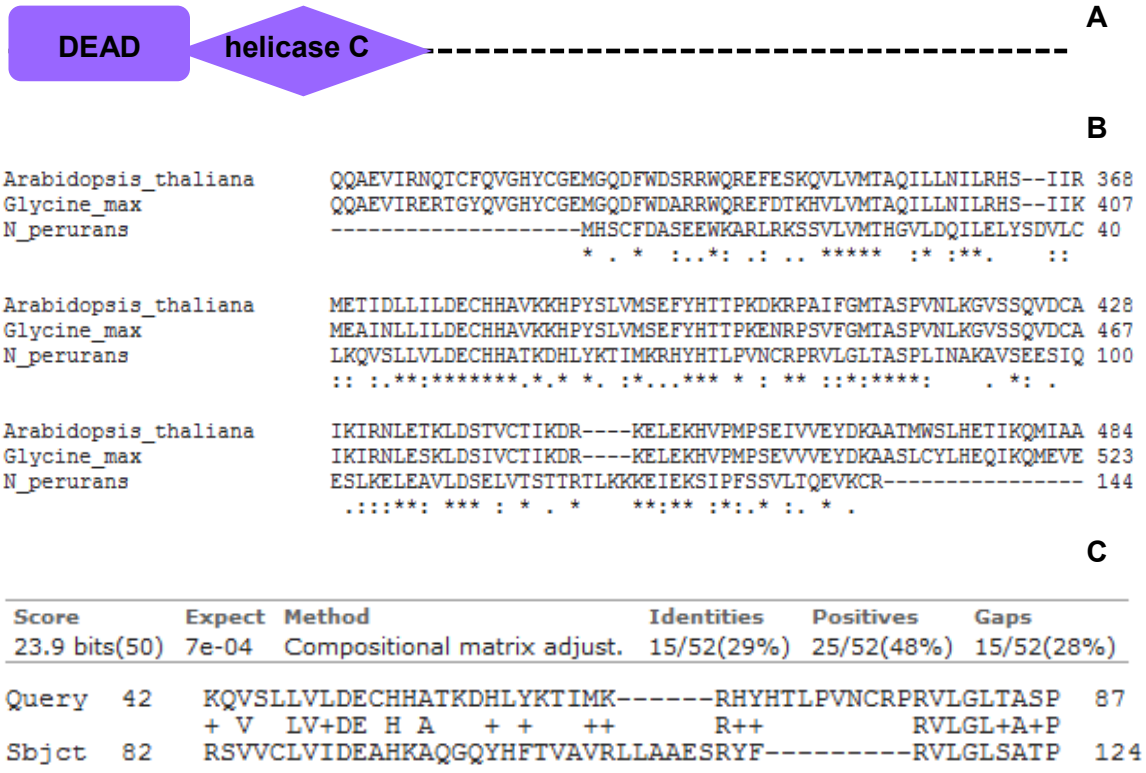

Figure 6.2 Candidate sharing significant level of homology with DEAD-like helicase (DEXDc) of Dicer. (A) Schematic representation of the ORF containing the DEXDc domains. Dotted lines indicate regions of unknown sequence. (B) Multiple sequence alignment of amino acid queries and their corresponding best-scoring hits. The bottom rows indicate the degree of conservation seen in the alignment column: (*) strictly conserved, (:)

highly conserved or (.) moderately conserved. (C) BLAST alignment of both *N. perurans* and *N. pemaquidensis* DEXDc candidates and related scoring results.

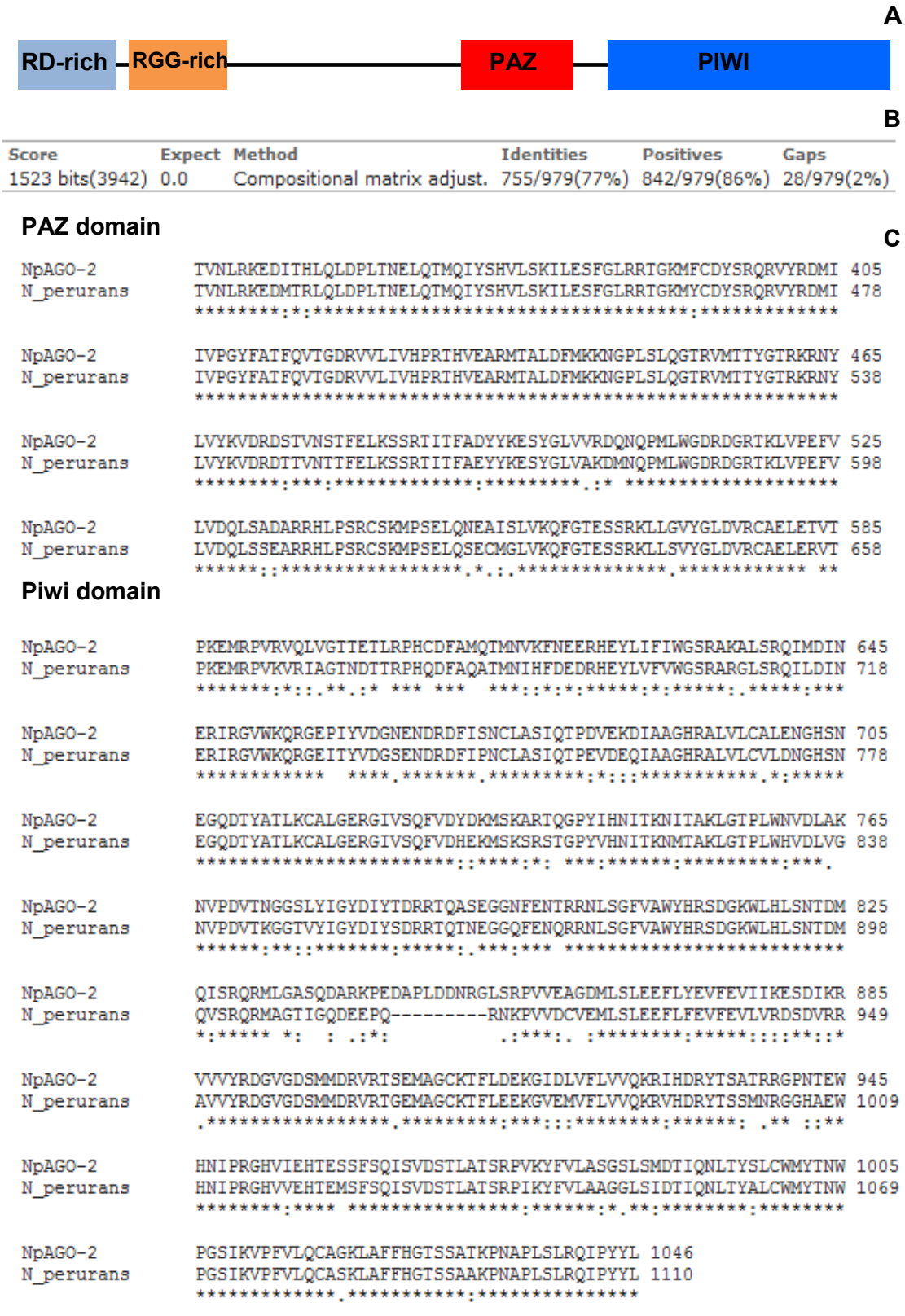

Figure 6.3 Candidate sharing high levels of homology with proteins from the Ago subfamily. (A) Schematic representation of the detected conserved domains. Solid lines

indicate sequences with no recognizable domains. (B) BLASTp sequence alignment score between Ago candidate 1 and *Np*AGO-2. (C) Multiple sequence alignment between the two protein candidates. The bottom rows indicate the degree of conservation seen in the alignment column: (*) strictly conserved, (:) highly conserved or (.) moderately conserved.

Figure 6.4 Candidate sharing high levels of homology with proteins from the Ago subfamily. (A) Schematic representation of overlapping ORFs containing conserved

domain. Solid lines indicate sequences with no recognizable domains. (B and C) Multiple sequence alignment and corresponding scores between *Np*Piwil-2 and Ago candidate 2 ORFs. The bottom rows indicate the degree of conservation seen in the alignment column: (*) strictly conserved, (:) highly conserved or (.) moderately conserved. Overlapped regions are represented in orange.

The activation of *N. perurans* Dicer and Ago candidates following the administration of RNAi duplexes haven't been validated yet. Nevertheless, the presence of an active Dicer was evidenced by the RNAse III assay which showed similar results than what was observed for *N. pemaquidensis*. dsRNA was completely degraded under incubation with *N. perurans* protein lysate (Figure 6.5, lane C and D), with digestion efficiency being reduced when serially diluted lysates were added to the substrate (Figure 6.5, lane E-H). Additionally, treating the amoebae lysate with 10x EDTA prior dsRNA incubation resulted on complete inactivation of Dicer-like enzymatic activity (Figure 6.5, lane I). However, despite the evidence revealed above, future efforts need to be directed towards obtaining the full length of the DEXDc-containing sequence and, therefore, validate if other conserved domains commonly found in Dicer are present in the protein candidate.

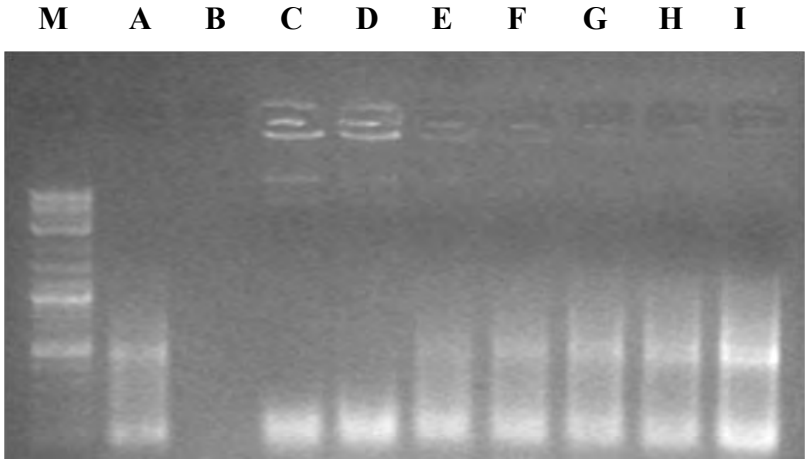

Figure 6.5 Agarose gel demonstrating the RNAse III activity assay. (A) 2 µg of dsRNA, (B) 2 µg of dsRNA incubated with commercially available *E. coli* RNAse III, (C-G) 2 µg dsRNA incubated with serially diluted *N. perurans* lysate (1:50, starting at 5 µg), (H), 2 µg of dsRNA incubated with buffer lacking the amoeba lysate, (I) 2 µg of dsRNA incubated

with *N. perurans* lysate (5 µg), previously inactivated with 10x EDTA. Incubation period of 1 h was adopted for all treatments. (M) 2-Log DNA marker (0.1-10 kb).

No assays were performed thus far aiming to validate the involvement of either *N. perurans* Ago candidates in RNAi mechanisms. However, considering *per*AGOl-2 shared significant level of homology with *Np*AGO-2, as well as displayed a N-terminal domain containing RGG repeats, which has been reported to be functionally relevant for RNAi-mediated degradation of mRNA [292], we suggest that such candidate could possibly be involved in the siRNA-mediated mRNA degradation.

An analogous supposition can be made for *per*Pikil-2, since such protein showed significant identity scores against *Np*Pikil-2 and other members of the Piwi clade, which could be an indication that *N. perurans* has retained key genes involved in the piRNA pathway. However, further functional studies are required to corroborate the above hypothesis.

6.3. Using the *Entamoeba histolytica* U6 promoter to drive the expression of short hairpin RNA in *N. perurans*

RNAi-mediated knockdown can be achieved by the administration of several RNAi duplexes, which are designed to mimic the exogenous and endogenous triggers of siRNA and miRNA pathways [341]. While some molecules, such as long dsRNA [342], small hairpin RNAs (shRNA) [343] and precursor miRNA (pre-miRNA) [344], activate the RNAi machinery by being recognized and processed into siRNAs by the ribonuclease III enzyme Dicer, synthetic siRNAs [345] and miRNAs [346] are directly incorporated into the RISC, serving as a guide to select fully complementary mRNA substrates for degradation.

Among the RNAi triggers, the development of DNA vector-based shRNA expression system appears as a simple and cost-effective method for inhibiting gene activity in either inheritable or inducible manner [347]. Expression of shRNAs in cells is typically accomplished by delivery of plasmid [234] or viral-based [348] vector systems. Once integrated into the host genome, the vector is transcribed in the nucleus into stem-loop hairpin structures consisting of paired antisense and sense strands (19-25 bp) connected by a loop of unpaired nucleotides (4-10 bp) [349]. The shRNA are then transported to the cytoplasm, where they are cleaved by Dicer to generate active siRNAs [350].

Selection of an appropriate promoter to drive the shRNA expression is one of the most important factors determining the knockdown efficiency mediated by DNA-based shRNAs [234]. In this context, small nuclear RNA U6 (U6) polymerase III (pol III) promoters are the most frequently employed for shRNA expression, as they naturally direct the synthesis of small, highly abundant noncoding RNA transcripts and have well defined sites of transcription and termination [351, 352].

To advance the use of RNAi tool for functional genomic research and future development of alternative treatment strategies against AGD of farmed Atlantic salmon (*Salmo salar*), we developed a 19 bp shRNA expression vector featuring the *Entamoeba histolytica* U6 promoter (*Eh*U6p). This promoter has been successfully employed by Linford et al. [63] to drive the expression of 29 bp plasmid-base shRNAs targeting *E. histolytica* endogenous genes. However, unlike the two-step PCR strategy used by Linford et al. [63], *Eh*U6p was incorporated into the plasmid vector via the one-step PCR method described at [343]. The ability of *Eh*U6p to drive the expression of shRNA in *N. perurans* was assessed by targeting and silencing the enhanced green fluorescent protein (EGFP).

6.3.1. Preparation of the *Entamoeba histolytica* U6 promoter

An aliquot of plasmid DNA containing the RNA polymerase III promoter of the *E. histolytica* U6 gene (GeneBank: U43841) was kindly provided by Dr Alicia Lindford (Department of Microbiology, University of Virginia). The 333 bp product was amplified using *Pfu* DNA polymerase (Promega) in PCR reaction containing 10x *Pfu* Buffer (Promega), 10 mM dNTPs (Invitrogen) and forward and reverse primers designed to cover the entire *Eh*U6p sequence (Table 6.1). The right size product was visualized on agarose gel and purified using the QIAquick PCR purification kit (Qiagen). The purified PCR fragment was then cloned into pGEM®-T Easy (Promega) and transformed into α-select gold efficiency competent cells (Bioline). Cloned inserts were sequenced in both directions using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the *Eh*U6p sequence confirmed using ChromasPro v.1.5 (Technelysium Pty. Ltd.).

6.3.2. Plasmid-based shRNA expression vector

The cloned *Eh*U6p was subsequently used as a template to construct EGFP (*Eh*U6p.shEGFP) and luciferase (*Eh*U6p.shLuc) shRNA expression plasmids, using the one-step PCR approach described by Wise et al. [343]. PCR was carried out using the forward primer previously employed to amplify the *Eh*U6p and a reverse primer designed to comprise the last 20 bp of the *Eh*U6p, target shRNA sense, loop, target shRNA antisense, stop and terminator sequences followed by a *Bam*H1 recognition site (Table 6.1 and Figure 6.6). The selected EGFP sequence

(GCTGACCCTGAAGTTCATC) was previously used by Kim and Rossi [353], while the luciferase (GCGGAATACTTCGAAATGT) target site was determined by using the Invitrogen BLOCK-iT™ RNAi Designer webpage (http://rnaidesigner.invitrogen.com/rnaiexpress/).

Table 6.1 Primer sequences used for construction of *Eh*U6p-driven shRNAs

primer	nucleotide sequences (5'-3')
*Eh*U6p.5'	TGTTTTTATGAAAAAGTGTATTTGG
*Eh*U6p.3'	CAATTTTATTTTTCTTTTTATCC
GFP.shRNA.3'	GGATCCTTCCAAAAAAGCTGACCCTGAAGTTCATCTCTCTTGAAGATA TGAACTTCAGGGTCAGCCAATTTTATTTTTCTTTT
Luc.shRNA.3'	GGATCCTTCCAAAAAAGCGGAATACTTCGAAATGTTCTTACTTGAAAC ATTTCGAAGTATTCCGCCAATTTTATTTTTCTTTT

PCR conditions for generating the shRNA expression vector were performed according to the second PCR step used by Lindford et al. [63] to develop a 29 bp shRNA using the same promoter: initial denaturation step at 95°C for 8 min;10 cycles of 95°C for 45 s, 18.5°C for 1:30 min and 68°C for 1:30 min; extra 30 cycles of 95°C for 45 s, 55°C for 1 min and 68°C for 1:30 min; and a final extension step for 5 min at 68°C. The PCR products were verified by gel electrophoresis and the correct size bands (~400bp) gel purified using QIAquick gel extraction kit (Qiagen). Following ligation (pGEM®-T Easy), recombinant plasmids containing perfect *Eh*U6p and shRNA nucleotide sequences were confirmed by sequencing prior to amoeba transfection.

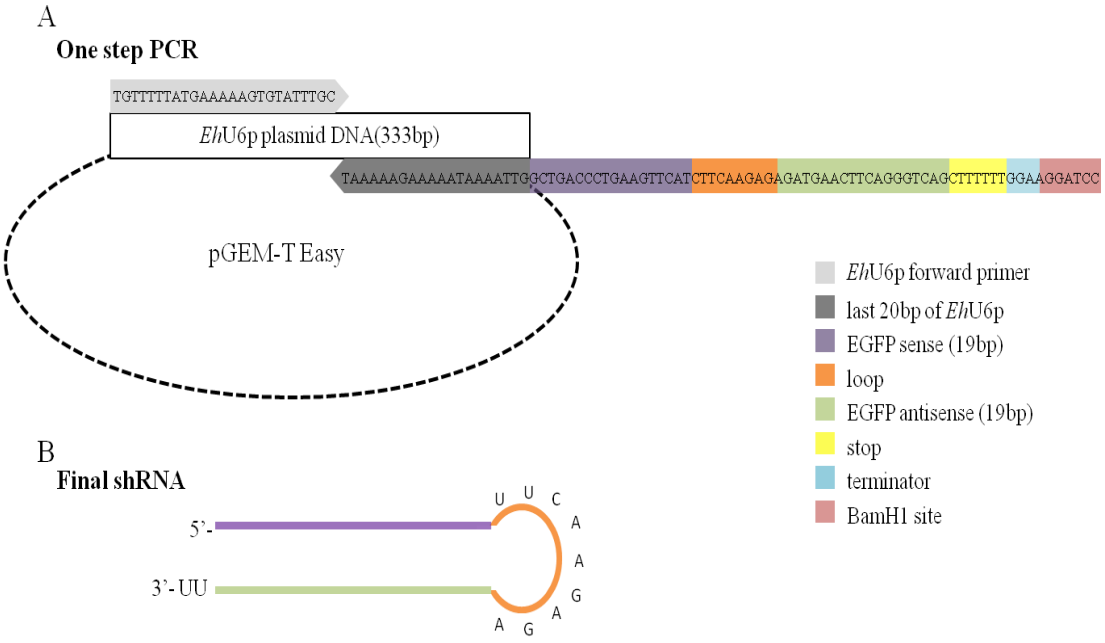

Figure 6.6 Diagram demonstrating the one-step PCR method employed to produce shRNA expression vectors. (A) *Eh*U6p plasmid DNA was used as a template in a PCR reaction containing a forward primer targeting the first 26 bp of *Eh*U6p (light gray) and a reverse primer covering the last the last 20 bp of the *Eh*U6p (dark grey), target shRNA sense (purple), loop (orange), target shRNA antisense (green), stop (yellow), terminator sequence (blue) and *Bam*H1 site (pink). (B) Schematic representation of final shRNA expression vector.

6.3.3. Transfection and knockdown validation by fluorescent microscopy

Transfection of shRNAs was carried out using Lipofectamine® RNAiMAX reagent (Invitrogen) and Opti-MEM® I Reduced Serum Medium (Invitrogen), according to the manufacturer's instructions. For fluorescence microscopy, pEGFP-N1 (Clonetech) was used to express EGFP in the amoeba cells and, therefore, to validate EGFP downregulation by the administration of shRNA. For this purpose, 500 ng of shRNA encoding the EGFP sequence (*Eh*U6p.shEGFP) or the irrelevant control (*Eh*U6p.shLuc) were co-transfected with 1 µg of pEGFP-N1. Transfection of

pEGFP-N1 alone (1 µg) was also performed as control. At 48 h post-transfection, trophozoites were monitored for EGFP expression using a Zeiss Axio Observer inverted microscope under EGFP filter set (excitation 430nm, emission 520 nm).

Despite using higher shRNA and pEGFP-N1 concentrations than in studies performed on vertebrates cell lines [343, 354], the EGFP fluorescent signal was detectable but not strong (Figure 6.7). Unexpectedly, fluorescence emission was observed is all treatment groups, despite with slight less intensity in *Eh*U6p.shEGFP treated trophozoites (Figure 6.7C).

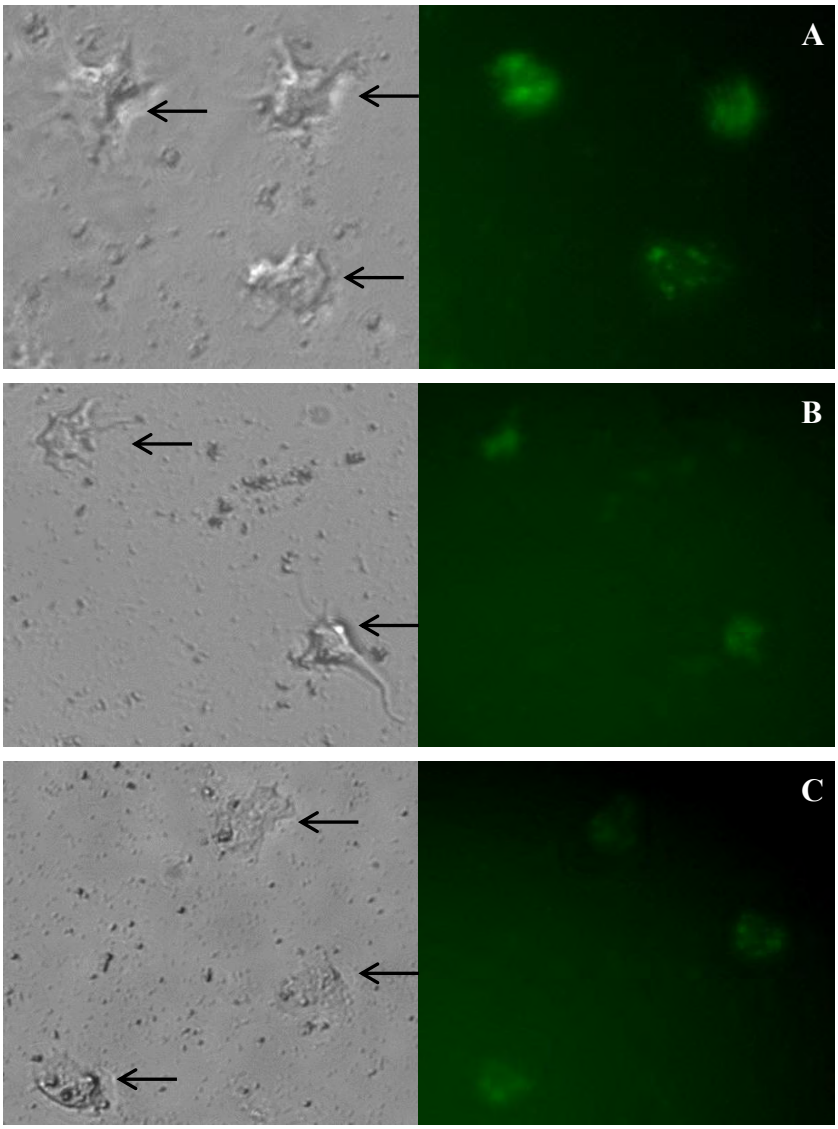

Figure 6.7 Validation of EGFP expression by fluorescence microscopy (40xmagnif). The figures show phase contrast and fluorescent image of trophozoites transfected with (A) pEGFP-N1 alone (B) *Eh*U6p.shLuc plus pEGFP-N1 (C) *Eh*U6p.shEGFP plus pEGFP-N1.Trophozoites are indicated by arrows.

As quantitative evaluation of the EGFP mRNA transcripts was not performed yet, it is still early to conclude whether pEGFP-N1 was processed by the amoeba RNAi machinery. Therefore, further transfection optimisation is required to enhance the EGFP fluorescent signal. One possibility would be to bring down the water salinity as the presence of different kinds of salts has been reported to reduce transfection efficiency [355]. Additionally, as it is unsure whether the *Eh*U6p will be recognised and expressed by *N. perurans,* the identification of a native promoter would be ideal for future development of RNAi strategies in the species.

6.4. Conclusions

The current chapter demonstrates that *N. perurans* trophozoites can be successful cultivated under *in vitro* conditions, as well as providing the first evidence that RNAi-associated genes have been evolutionarily retained in the causative agent of AGD. Despite being promising, these results are still preliminary and the reality of applying RNAi technology to develop new treatment strategies against AGD still distant. Unfortunately, the attempts to employ *Eh*U6p-driven shRNA to confirm the existence of a functional RNAi pathway in *N. perurans* haven't succeeded yet. Therefore, more research efforts have to be devoted to this in order to fully elucidate the RNAi mechanisms in the species.

CHAPTER 7: General Discussion

RNAi is a naturally occurring mechanism of gene regulation that is thought to have evolved from an early defense mechanism against viruses [356]. The presence of dsRNA is recognized as foreign and is destroyed with any single stranded RNA, including mRNA of the same sequence. Degradation of mRNA results in the post transcriptional inhibition of gene expression and the prevention of protein synthesis [357]. This natural mechanism for sequence-specific gene silencing promises to revolutionize experimental biology and may have important practical applications in functional genomics and development of new treatment strategies against pathogens of economically important aquaculture species. With regards to AGD of Atlantic salmon, RNAi-based technology could greatly assist in the validation of substitute treatment approaches to the conventional freshwater bathing method.

In the present thesis a combination of bioinformatics and experimental assays were employed to provide foundational evidence that the RNAi pathway has been evolutionarily retained in species from the *Neoparamoeba* genus.

7.1. Identification of putative proteins encoding conserved domains of Dicer and Ago

In the current study, *N. perurans* and *N. pemaquidensis* annotated transcriptome databases were employed to identify putative sequences containing conserved domains found in Dicer and Ago families. Homology analyses of translated transcripts revealed that neither species contain a putative candidate presenting all the predicted domains of Dicer. Nevertheless, a potential candidate comprising the Dicer DEXD helicase motif was identified in both databases

(*Np*DEXDc and *per*DEXDc, Chapter 3 and 6). Despite sharing significant similarity against Dicer of several species, BLASTp alignment of both DEXDc sequences showed that these proteins are only slightly similar to one another (29% identity, 7^{e-4}).

Interestingly, pairwise alignment of each DNA sequence against the DEXD helicase motif of both *Neagleria gruberi* Dicer-like proteins (EFC50206.1 and EFC44666.1) revealed that while *per*DEXDc is highly related with EFC50206.1 (38% identity, 1^{e-18}), *Np*DEXDc shares significant level of homology against EFC44666.1 (50% identity, 2^{e-10}). Unfortunately, no studies have been addressed to investigate the involvement of either *N. gruberi* Dicer-like genes in the RNAi pathway. Such information would greatly assist on further characterization of *Neoparamoeba* Dicer candidates, as *N. gruberi* is the only amoeba species that contains putative amino acid (aa) sequences covering all the expected conserved motifs of Dicer. Despite that, a functional assay demonstrated that *N. pemaquidensis* DEXDc candidate was significantly up-regulated following the administration of *in vitro* transcribed dsRNA, suggesting its involvement in the siRNA-mediated gene silencing pathway. The same experiment has not yet been replicated for *N. perurans*, preventing any assumption on the candidate's participation in RNAi-related mechanisms. However, considering that complete dsRNA degradation was observed following incubation with a crude protein lysate of both amoeba species, it is suggestive that an active Dicer in also present in *N. perurans*. Nevertheless, the observed Dicer-mediated degradation should be further validated by reproducing the RNAse III activity assay using purified recombinant protein of the Dicer candidates.

Apart from the single contig covering the DEXDc, another partial aa sequence containing a RNAse III C- terminal domain (RIBOc) was identified in the *N. pemaquidensis* database (*Np*Drosha, Chapter 3). However, the candidate showed

significant identity homology to Drosha, a Dicer-like RNAse III enzyme involved in the miRNA-pathway. The hypothesis that the putative candidate gene is not involved in the classical RNAi pathway was supported by qRT-PCR analysis, where the transcript mRNA levels were not influenced by the presence of dsRNA. Additionally, the candidate did not share significant level of homology against the only *Entamoeba histolytica* Dicer candidate (EAL45114), which despite of a containing just an RNAse III domain has been proved to be functionally involved in dsRNA cleavage [285].

Database searches also revealed multiple translated reading frames encoding conserved regions of the Ago superfamily. While four putative proteins were identified for *N. pemaquidensis*, two covering both PAZ and Piwi (*Np*AGO-2 and *Np*Piwil-2) and other two partial sequences sharing significant homology with the Piwi domain (*Np*Piwil-1 and *Np*PiwiS), two candidates containing both Ago motifs were detected in the *N. perurans* database (*per*AGOl-2 and *per*Piwil-2). Sequence alignment revealed that both AGO-like (*Np*AGO-2 vs. *per*AGOl-2) and Piwi-like (*Np*Piwil-2 vs. *per*Piwil-2) candidates are highly similar, suggesting that they are orthologs of each other.

Similar to both DEXDc-containing aa sequences, *Np*AGO-2 and *per*AGOl-2 appear to be homologous (22% identity, 8^{e-29} and 54% identity, 1^{e-29}) to the *N. gruberi* Ago (EFC41986.1). Therefore, considering the high abundance of RGG repeats towards the N-terminal domain of each AGO-like putative protein, as well as that *Np*AGO-2 was the only *N. pemaquidensis* Ago candidate significantly up-regulated by the administration of siRNA, we propose that both candidates are involved in siRNA-mediated mRNA degradation. Conversely, candidates highly related to members of the Piwi clade (*Np*Piwil-1, *Np*Piwil-2 and *per*Piwil-2) are expected to have a role in the Piwi-pathway. Moreover, it also appears that *N.*

pemaquidensis has retained an ortholog (*Np*PiwiS) to the new class of Piwi-containing proteins found in tripanosomatids species: the Piwi "solo" (PiwiS) [293]. However, since these proteins are found in both RNAi positive and negative species, there is still no evidence of their involvement in any of the small RNA-related mechanism [56].

Therefore, the results suggest that *Neoparamoeba* spp. has retained key genes involved not only in the RNAi machinery, but also in the miRNA and piRNA pathways. The fact that both DEXDc and Ago-like candidates of either species showed high level of homology against Dicer and Ago of an RNAi positive amoeba species (*N. gruberi*) is further evidence of the above hypothesis. However, efforts still need to be directed towards obtaining the full sequence of the ORFs of interest, as well as to confirm the role of each putative candidate in the related small RNA-mediated pathway.

7.2. Functional evidence for the presence of active RNAi machinery in *Neoparamoeba* spp.

Further evidence for the presence of functional RNAi machinery in *Neoparamoeba* spp. was supported by gene silencing experiments using different RNAi trigger molecules, such as: bacterially expressed dsRNA, *in vitro* transcribed dsRNA, esiRNA pool and shRNA. As the *in vitro* culture of *N. perurans* was only validated towards the end my PhD candidature, all the experiments, apart from the one using shRNA, were performed with *N. pemaquidensis*.

Bacteria expressing dsRNA targeting *N. pemaquidensis* specific genes (β-actin and EF1-α) was either directly fed to the amoeba or submitted to a purification step and administered via immersion at 2, 20 and 50 µg/mL. As amoebae naturally

feed on bacteria, the administration of dsRNA through bacterial ingestion would provide a direct, continuous and cost-effective RNAi delivery system. However, the results showed that RNAi delivery via ingestion triggered a delayed and less effective silencing response than immersion. Additionally, while soaking allows equal exposure of trophozoite to the dsRNA solution, oral administration results in uneven dsRNA uptake. As a result, the precise delivery dose cannot be estimated, preventing an accurate assessment of the amount of ingested bacteria required to trigger the RNAi machinery in the species. Direct ingestion of dsRNA-expressing bacteria also resulted in target-dependent knockdown as only β-actin mRNA levels were significantly suppressed.

Unlike feeding, delivery of purified bacterially expressed dsRNA via immersion promoted successful downregulation of both target genes. However, a more efficient silencing effect was observed for β-actin. The inhibition extent and persistence was also dose-dependent and daily dsRNA administration improved knockdown efficacy of EF1-α, but not of β-actin. Surprisingly, *in vitro* transcribed dsRNA designed against the same target genes regions failed to trigger significant mRNA suppression when delivered by soaking. However, as a slight reduction of both target genes mRNA levels was detected at 24 h, it is expected that the observed downregulation could be enhanced by either administration of higher dsRNA doses or through direct transfection. Therefore, further optimization is required to ensure that *in vitro* transcribed dsRNA can elicit successful silencing effect in *N. pemaquidensis*.

The above assumption is supported by the esiRNA-mediated gene silencing experiments, which showed that transfected amoebae presented higher knockdown efficiency than those treated with the same transfection mix lacking lipofectamine. Similarly, trophozites transfected with a siRNA fluorescent control displayed

considerably stronger fluorescent signal as opposed to the ones simply soaked with the same RNAi duplexes. This suggests that the knockdown effectiveness was directly associated with the amount of esiRNAs available in the amoeba cytoplasm.

Microscopy analysis also demonstrated that administration of bacterially expressed dsRNA distinctly affected cell morphology of treated amoeba, when delivered by immersion. Unexpectedly, β-actin suppressed trophozoites developed into a dormant cyst-like stage, which has not been previously reported in this species. However, similar effect was not detected when the same target gene was silenced by other RNAi molecules, suggesting the observed phenotype was not related to the target gene downregulation. Nevertheless, considering the administration of bacterially expressed dsRNA targeting luciferase and EF1-α was not able to elicit the same phenotypic change, we believe that the morphological response described above was not associated to a toxic effect caused by the bacterial dsRNA exposure. Therefore, further studies are required in order to better understand such occurrence.

With regards to *N. perurans*, a 19 bp shRNA driven by the *Entamoeba histolytica* U6 promoter was employed in the attempt to validate the existence of functional RNAi machinery in the species. For this purpose, the ability of *N. perurans* to express the foreigner promoter was evaluated by fluorescent microscopy using the exogenously expressed EGFP. Unexpectedly, fluorescence emission was observed is all treatment groups, despite being slightly less intense in trophozoites transfected with shRNA targeting EGFP. However, as the effectiveness to express shRNA molecules is known to vary among promoters [358], a stronger fluorescent signal is required in order to accurately assess potential partial suppression. While quantitative evaluation of EGFP mRNA transcripts through qRT-PCR could answer the question, the establishment of an effective fluorescence-based system would allow quicker and more affordable assessment of knockdown efficiency, especially

for optimisation purposes. Therefore, further attempts are required in order to improve the EGFP fluorescent signal in *N. perurans*. Additionally, the identification of a native promoter would possibly enhance the possibilities of achieving effective shRNA expression by the amoeba. Consequently, it is still early to suggest that RNAi-mediated knockdown can be successful achieved in *N. perurans*.

7.3. Final conclusions and future directions

In summary, this thesis has provided strong evidence that both infective and non-infective species from *Neoparamoeba* spp. have evolutionarily retained key genes involved not only in the canonical RNAi mechanism, but also in the miRNA and piRNA pathways. The presence of functional RNAi machinery in the genus was also validated by gene silencing experiments. However, knockdown efficiency has proven to be dependent on the target gene, delivery strategy, dosage, administration frequency and RNAi triggers. Despite being promising, the results presented in the current study are still preliminary and the reality of employing RNAi-based technology in the development of alternative treatment strategies against AGD requires further work. Hence, fundamental research efforts are still required to obtain a complete picture of RNAi mechanisms in *Neoparamoeba* species. Furthermore, the development of a suitable *in vivo* fish model system to investigate the effect of gene-specific knockdown on amoeba-host interaction would be also highly desirable. This would contribute significantly to a better understanding of the complex mechanism involved in amoeba attachment, as well as to validate the efficacy of potential treatment candidates. In addition, the possibility of using RNAi technology to unravel the mystery behind loss of virulence in certain *Neoparamoeba* strains could be of great benefit, especially now that transcriptome databases for both infective and non-infective species are available. Overall, the outcome of this thesis has provided a

solid foundation for further investigation of the application of RNAi-based approaches against AGD in farmed Atlantic salmon.

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APPENDIX:

List of sequences:

>β-actin
ATGTGCGACGACGAAGTTCAAGCTCTTGTGGTGGACAATGGGTCCGGTATGTGCAAAGCCGGATTCGC
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TCGGTATGGACACCAAGGACTCCTTCGTCGGAGACGAGGCCCAATCCAAGCGTGGTATCCTCACCTTG
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CCGGGCGTGATTTGACTGACTACTTGATGAAGATCTTGACCGAGCGTGGGTACTCCTTCACCACCACC
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GATGCAGACCGCTGCCTCTTCCTCTGCCCTCGAGAAGTCCTA

>EF1-α
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>Luciferase
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