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.....”

TABLE OF CONTENTS:

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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STATEMENT OF THE CONTRIBUTIONS OF JOINTLY AUTHORED PAPERS:

Literature Review:

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)

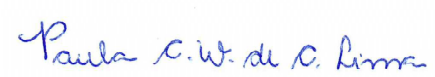
CHAPTER 5

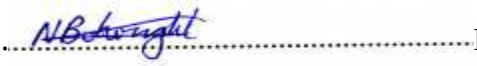
Lima, PC; Botwright, NA; Harris, JO; Cook, M. Ingestion of bacterially expressed dsRNA induces target gene-dependent knockdown in species from the genus *Neoparamoeba* (submitted to Aquaculture)

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.


..... Lima, P.C


.....Botwright, N.A.

..... Harris, J.O.


..... Cook M.

LIST OF FIGURES:

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

LIST OF TABLES:

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 6