A Functional Study of Major Histocompatibility Expression and Immune Function in Rainbow Trout, (*Oncorhynchus mykiss*)

by

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ABSTRACT

Major Histocompatibility Complex (MHC) receptors serve a critical role in self/non-self recognition through the presentation of peptide antigen to circulating T lymphocytes and are also believed to play a role in mate selection. Through the development of antibodies to MHC homologues in trout, this report demonstrates the presence of MHC expression in germ cells, as well as a soluble form in seminal fluid. What role these immune molecules may perform in reproduction and mate selection is discussed. In addition, as ectotherms, fish are often subjected to low temperatures. Previous data indicates that the expression of these genes is abolished by low temperatures. Employing these same antibodies, this report further demonstrates that trout maintain the expression of MH I and its critical light chain component, beta-$_2$-microglobulin when subjected to 2°C for 10 days. Expression of the MH II receptor sub-units however, was sensitive to both confinement stress and low-temperature *in vivo*, as well as to factors secreted from a known fungal pathogen in cultured macrophage. As the cause of "winter kill", Saprolegniales cultures induced homotypic aggregation and pro-inflammatory gene expression in the macrophage cell line, RTS11 as well as down-regulation of MH II. Though no evidence of fungal toxins was evident, fungal spore size appeared to exceed macrophage phagocytic capabilities. Taken together, such a loss of MH II expression at low temperature may allow for establishment of fungal and bacterial diseases and that upon the return to warmer temperatures, saprolegniales have the ability to maintain MH II down-regulation and evade immune recognition. Concurrent to the study of MH expression, this report includes the first cloning and characterization of calreticulin (CRT) in fish. Like its mammalian homologue and primary chaperone to MHC receptors and other immune proteins, trout CRT appears to be a single copy gene with ubiquitous tissue distribution, displaying anomalous migration as a doublet with relative molecular mass of 60kD. Despite its promoter containing endoplasmic reticulum stress elements (ERSE), trout CRT expression did not increase upon treatment with several calcium homeostasis antagonists. Treatment of peripheral blood leukocytes with phytohemaglutinin did reveal a qualitative increase in cell surface expression, as seen in mammals; however, cellular protein levels did not change, suggesting that, in trout, CRT function may be regulated through cellular sub-localization, rather than through changes in gene expression, as it is in mammals.

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Lastly, to my family, who's love and support has allowed me to continue; to Wendy & Maddigan for their love, patience and support.

DEDICATION

For my family; my dad who taught me to be a father, my mom who taught me to love, and all those that I love and for everything you do.

In loving memory of my grandmother, Hilda Marian Winstanly Purton.
May 17, 1913 - August 8, 2006

TABLE OF CONTENTS

ABSTRACT III
ACKNOWLEDGEMENTS IV
DEDICATION V
TABLE OF CONTENTS VI
LIST OF TABLES IX
LIST OF FIGURES X
LIST OF ABBREVIATIONS XII

CHAPTER 1 GENERAL INTRODUCTION 1
GENERAL INTRODUCTION 2
1.1. THE MAJOR HISTOCOMPATABILITY COMPLEX 2
1.1.1. The Class I Major Histcompatibility Receptor 2
1.1.2. Calreticulin 3
1.1.3. The Class II Major Histocompatability Receptor 3
1.1.4. MHC & Mate Choice 5
1.1.5. Major Histocompatibility Receptors in Fish 5
REFERENCES 7

CHAPTER 2 KEEPING THEIR COOL: BETA-$_2$-MICROGLOBULIN GENE EXPRESSION IS MAINTAINED IN RAINBOW TROUT AND ATLANTIC SALMON KEPT AT LOW TEMPERATURES. 10
ABSTRACT 11
2.1. INTRODUCTION 12
2.2. MATERIALS & METHODS 13
2.2.1. Fish 13
2.2.2. Northern blot analysis of Atlantic salmon tissues 14
2.2.3. RT-PCR analysis of rainbow trout tissues 15
2.2.4. Cloning and development of salmonid β_2m polyclonal antibodies. 16
2.2.5. Western blot analysis of Atlantic salmon and rainbow trout tissues 17
2.2.6. In vitro cell challenge 18
2.2.7. Indirect epifluorescent detection of surface antigens 18
2.3. RESULTS 18
2.3.1. β_2m transcript at low temperature 18
2.3.2. Development of antibodies recognizing trout β_2m 20
2.3.3. β_2m cellular protein expression at low temperature 23
2.3.4. Surface β_2m expression at low temperature 26
2.4. DISCUSSION 26
REFERENCES 30

CHAPTER 3 FINDING A CHAPERONE: MOLECULAR CLONING AND CHARACTERIZATION OF CALRETICULIN FROM RAINBOW TROUT, (*ONCORHYNCHUS MYKISS*)....... 32
ABSTRACT 33
3.1. BRIEF COMMUNICATION 34
REFERENCES 43

CHAPTER 4 CALRETICULIN IN RAINBOW TROUT: A LIMITED RESPONSE TO ER STRESS....... 46
ABSTRACT 47
4.1. INTRODUCTION 48
4.1.1. Calreticulin in mammals 48
4.1.2. Calreticulin in fish 50
4.2. MATERIALS AND METHODS 51
4.2.1. Cloning of Trout Calreticulin Promoter Sequence 51
4.2.2. Production of recombinant trout CRT 51
4.2.3. Development of Trout Calreticulin Antisera 52
4.2.4. Rainbow trout tissue & cell preparations for in vitro challenge 53
4.2.5. RT PCR analysis of Trout Calreticulin Transcript 53
4.2.6. Western Blot Analysis 54
4.2.7. De-glycosylation 54
4.2.8. Indirect epifluorescent detection of surface CRT 55
4.3. RESULTS 56
4.3.1. Protein Expression & Antibody development 56
4.3.2. Cloning of rainbow trout calreticulin promoter region 56
4.3.3. In vitro challenge & trout calreticulin steady state levels in primary cultured cells ... 60
4.3.4. In vitro CHA challenge in a stable trout cell line 62
4.3.5. CRT surface expression of cultured trout PBL 62
4.4. DISCUSSION 66
REFERENCES 70

CHAPTER 5 RESPONSE OF THE RAINBOW TROUT MONOCYTE/MACROPHAGE CELL LINE, RTS11 TO THE WATER MOLDS *ACHLYA* & *SAPROLEGNIA* 75
ABSTRACT 76
5.1. INTRODUCTION 77
5.2. MATERIALS AND METHODS 79
5.2.1. Fungal cultures 79
5.2.2. Calcofluor staining of fungal inocula 79
5.2.3. Cell culture 79
5.2.4. In vitro challenge 80
5.2.5. Microscopic examination and recording 80
5.2.6. Reverse transcriptase (RT) PCR analysis of gene transcripts 80
5.2.7. Western blot analysis of cellular protein expression 82
5.3. RESULTS 83
5.3.1. Cellular effects of fungal-macrophage co-culture 83

5.3.2. *Effects of fungal co-culture on macrophage gene expression* ... 90
5.3.3. *Effects of fungal co-culture on macrophage protein expression* ... 92
5.4. DISCUSSION ... 95
5.4.1. *Cellular effects of fungal-macrophage co-culture* ... 95
5.4.2. *Effects of fungal co-culture on trout macrophage gene expression* ... 96
5.5. CONCLUSIONS ... 99
REFERENCES ... 100

CHAPTER 6 FISHING FOR A MATE: SALMONID MAJOR HISTOCOMPATIBILITY EXPRESSION IN REPRODUCTIVE TISSUES ... 105
ABSTRACT ... 106
6.1. INTRODUCTION ... 107
6.2. MATERIALS AND METHODS ... 109
6.2.1. *Fish* ... 109
6.2.2. *Production of recombinant trout MH I* ... 110
6.2.3. *Development of Trout MH I antisera* ... 111
6.2.4. *Western blot analysis* ... 111
6.2.5. *De-glycosylation* ... 112
6.2.6. *Purification of soluble MH I* ... 112
6.3. RESULTS ... 113
6.3.1. *Development of polyclonal antisera to recombinant trout MH I heavy chain* ... 113
6.3.2. *MH I expression among trout reproductive tissues & fluids* ... 117
6.3.3. *MH II expression in trout sperm and ovary* ... 120
6.4. DISCUSSION ... 120
REFERENCES ... 125

CHAPTER 7 OUT IN THE COLD: DISCUSSION, CONCLUSIONS AND FUTURE AIMS ... 128
7.1. GENERAL DISCUSSION ... 129
7.1.1. *Beta-$_2$-microglobulin* ... 129
7.1.2. *MH I* ... 130
7.1.3. *Calreticulin* ... 133
7.1.4. *MH II* ... 133
7.1.5. *Response of trout macrophage to a known fungal pathogen* ... 137
7.2. CONCLUSIONS ... 138
REFERENCES ... 140

LIST OF TABLES

Table 5-1. Oligonucleotide sequences employed for RT PCR analysis. 81

LIST OF FIGURES

Figure 1-1. The MHC I antigen presenation pathway. 4

Figure 2-1. β_2m transcript steady-state levels in PBL kept at 2°C. 19

Figure 2-2. Northern blot analysis of trout β_2m transcript in Atlantic salmon kept at 5 & 10°C. . 21

Figure 2- 3. Western blot analysis of anti-trout β_2m antiserum cross-reactivity. 22

Figure 2-4. Western blot analysis of β_2m protein in Atlantic salmon kept at 5 & 10°C. 24

Figure 2-5. Western blot analysis of β_2m protein in tissues of rainbow trout kept at 2°C. 25

Figure 2-6. Surface β_2m expression in PBL of rainbow trout kept at 2°C & 13°C. 27

Figure 3-1. Nucleotide and deduced amino acid sequence of rainbow trout calreticulin cDNA. . 36

Figure 3-2. Alignment of trout calreticulin with known amino acid sequences. 37

Figure 3-3. Phylogenetic analysis of known calreticulin nucleotide sequences. 39

Figure 3-4. Southern blot analysis and tissue distribution assay of trout calreticulin. 41

Figure 4-1 Tissue distribution of trout calreticulin protein expression. 57

Figure 4-2. Western blot analyses of trout gill lysate following *in vitro* deglycosylation. 58

Figure 4-3. Nucleotide sequence of putative trout CRT proximal promoter. 59

Figure 4-4. Effects of CHA on primary cultured trout PBL. 61

Figure 4-5. Effects of CHA on the rainbow trout macrophage cell line, RTS11. 63

Figure 4-6. Surface calreticulin protein expression in trout PBL. 64

Figure 4-7. Cellular calreticulin protein levels in PHA-treated PBL. 65

Figure 5-1. RTS11 adherence to cultures of *Achlya bisexualis* and *Saprolegnia parasitica*. 84

Figure 5-2. Calcofluor staining of fungal hyphae in trout macrophage co-culture. 85

Figure 5-3. RTS11 response to heat-killed *Achlya* mycelium. 86

Figure 5-4. Testing of adherence and aggregation by cell-type and substrate. 88

Figure 5-5. HA in RTS11 by *Achlya* cultures and their filtrate. 89

Figure 5-6. RTS11 gene expression in the presence of *Achlya*. 91

Figure 5-7. MH protein expression in the presence of *Achlya* & *Saprolegnia*. 93

Figure 5-8. RTS11 gene expression in the presence of heat-killed *Achlya*. 94

Figure 6-1. Antiserum specificity towards various fish gill lysates. 114

Figure 6-2. Western tissue distribution assay for anti-trout MH I reactivity. 115

Figure 6-3. Differential N-glycosylation of trout MH I. 116

Figure 6-4. MH I protein expression in trout reproductive tissues and their fluids. 118

Figure 6-5. Two-dimensional western blot analysis of purified serum MH I. 119
Figure 6-6. MH II expression detectable in trout sperm and ovary lysates. 121
Figure 7-1. MH I transcript and protein expression at 2°C... 131
Figure 7-2. Surface MH I expression in trout PBL at 2° & 13°C. 132
Figure 7-3. Western blot analysis of trout MH sub-units following *in vitro* de-glycosylation. .. 134
Figure 7-4. Trout MH II protein expression during low-temperature and confinement.............. 136

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
β_2m	beta-$_2$-microglobulin
cDNA	complementary DNA
CHA	calcium homeostasis antagonist
COX	cyclooxygenase
CRT	calreticulin
CIITA	class II transactivator
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
eEF1α	elongation factor alpha
ER	endoplasmic reticulum
ERSE	endoplasmic reticulum stress element
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
FBS	fetal bovine serum
gDNA	genomic DNA
GFP	green fluorescent protein
HA	homotypic aggregation
hsp	heat shock protein
IFN	interferon
Ig	immunoglobulin
IgG	immunoglobulin G
IgM	immunoglobulin M
Ii	invariant chain
IL	interleukin
Jak	Janus kinase
kD	kiloDalton
L-15	Leibovitz's media

LPS	lipopolysaccharide
MH	major histocompatibility
MHC	major histocompatibility complex
MH I	class I major histocompatibility receptor
MH II	class II major histocompatbility receptor
mRNA	messenger RNA
PBL	peripheral blood leukocyte
PCR	polymerase chain reaction
pI	isoelectric point
PHA	phytohemagluttinin
Poly I:C	poly inosinic:poly cytidylic acid
RNA	ribonucleic acid
RTG-2	a rainbow trout gonadal fibroblast-like cell line
RT-PCR	reverse transcriptase-PCR
RTS11	a rainbow trout spleen macrophage-like cell line
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
STAT	signal transducers and activators of transcription
TAP	transporter associated with peptide antigen
TNF-α	tumor necrosis factor- α

Chapter 1

General Introduction

GENERAL INTRODUCTION

The goal of this work was to provide new insights into the function and regulation of key components of adaptive immunity in fish by exploring the expression of the Major Histocompatibility and related genes in rainbow trout and Atlantic salmon. By utilizing these two valuable aquaculture species, this work may allow for further development of disease prevention protocols and perhaps improved fish stocks.

1.1. THE MAJOR HISTOCOMPATABILITY COMPLEX

1.1.1. The Class I Major Histcompatibility Receptor

It has been over fifty years since the first description of what has become known as the Major Histocompatibility Complex (MHC; reviewed by Klein, 1986). Since that time, the genes comprising this region have been identified throughout the jawed vertebrate lineage, including mammals, birds, amphibians and sharks (reviewed by Shand & Dixon, 2001). Comprising three regions designated class I, II & III, the MHC contains genes encoding immune & accessory molecules, which facilitate antigen presentation, as well as complement, heat shock proteins and several cytokines (Klein, 1986). Most notable; however, are the two cell-surface molecules that bear its name, the class I & II MHC receptors. The MHC I receptor is a cell-surface heterodimer comprising of a membrane-bound 45kD heavy "alpha" chain and a 12kD light chain, beta-$_2$-microglobulin (β_2m; Klein, 1986). Though not encoded within the MHC region, this light chain is critical to the cell-surface stability of this receptor (Rock *et al.*, 1991; Vitiello *et al.*, 1990). As a member of the Immunoglobulin (Ig) superfamily, the class I alpha chain is a glycoprotein containing three Ig domains, designated alpha 1, 2 & 3. The alpha 1 & 2 domains are highly polymorphic and form the peptide binding groove suitable for binding the diverse set of peptide antigens. Peptide antigens, typically of endogenous origin, representing cellular, as well as viral and tumour proteins, are derived through proteasomal degradation and translocated to the ER by TAP, the transporter associated with peptide antigen (Fig. 1-1; reviewed by Cresswell *et al.*, 1999).

1.1.2. Calreticulin

Several chaperones have been shown to associate and facilitate in the proper folding and peptide loading of the MHC I receptor during its association within the endoplasmic reticulum (ER; Cresswell *et al.*, 1999). In mammals, calreticulin (CRT) is considered a critical chaperone to glycoprotein folding, which includes most immune molecules, especially MHC (Michalak *et al.*, 1999). Originally identified through its capacity for calcium binding, calreticulin also serves as the primary modulator of ER calcium stores. Within the ER, CRT has been shown to displace calnexin, as the primary chaperone, following β_2m-MH I heterodimer formation (Fig. 1-1; Sadasivan *et al.*, 1996). This highly conserved protein has been identified in nearly all eukaryotes, including: animals, plants and more recently in slime molds (reviewed by Michalak *et al.*, 1999). Knock-out studies in mice have revealed that this protein plays a critical role in embryonic development (Maesali *et al.*, 1999). Disruption of ER calcium homeostasis, through the use of calcium ionophore, Ca^{++}-ATPase inhibitors, as well as heat shock and viral infection, have been shown to cause a dramatic up-regulation of CRT transcript and protein steady-state levels in mammals (Waser *et al*., 1997). The up-regulation of CRT is regulated through endoplasmic reticulum stress elements (ERSE) within its promoter, which are activated through the unfolded protein response (Yoshida *et al*., 2003).

1.1.3. The Class II Major Histocompatability Receptor

The MHC II receptor is also a heterodimeric molecule comprised of two membrane-bound sub-units, alpha and beta. Each having molecular masses of roughly 30kD, these two sub-units, with their highly polymorphic Ig domains, form a peptide binding groove similar to that of the class I receptor (not shown; Blair *et al.* 1995). The MHC II receptor presents exogenous peptide antigen *via* the lysosomal pathway and its expression is typically restricted to professional antigen presenting cells such as macrophage and dendritic cells, which phagocytose extracellular pathogens, including bacteria and fungi (Glimcher & Kara, 1992).

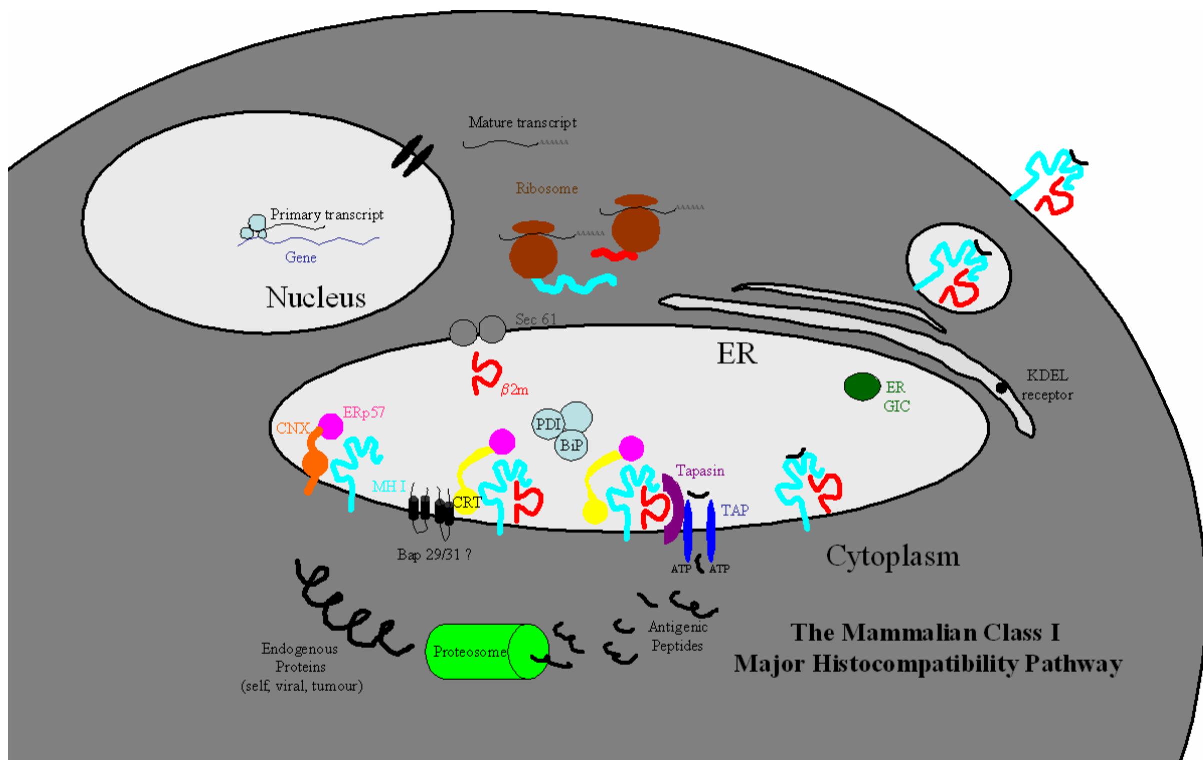

Figure 1-1. The MHC I antigen presenation pathway.

A schematic represenation of the MH I antigen presentation pathway based upon recent data. Briefly, nuclear MHC gene transcripts are translated to nascent polypeptides and translocated to the ER *via* Sec 61. Several ER chaperones, beginning with calnexin (CNX) and Erp57 facilitate proper folding of MH I and β_2m separately. Other accessory chaperones, including Bap29/31, PDI and BiP have also been shown to interact with various glycoproteins throughout their synthesis. Upon heterodimer formation, the membrane-bound CNX is displaced by the soluble calreticulin (CRT) as the primary chaperone and final association with TAP, mediated through tapasin, completes the formation of the peptide loading complex. Cytosolic peptides, generated through proteasomal degradation, are translocated by an ATP-dependent mechansim to the ER by TAP. Peptide loading of the MH I – β_2m heterodimer leads to chaperone release and receptor translocation to the cell surface. Gene transcript, translated protein and final cell surface transport therefore represent three levels of control of function.

Subsequent fusion of the endosomal compartment with the lysosome leads to break down of the foreign peptides as well as the invariant chain (Ii), an accessory molecule which temporarily occupies the peptide binding region of the MHC II receptor through its CLIP (Class II-associated Invariant chain Peptide) region (Romagnoli *et al.*, 1994). Lysosomal degradation of the invariant chain facilitates the displacement of the CLIP region for the newly digested foreign peptide and subsequent transport to the cell surface for presentation to circulating lymphocytes.

1.1.4. MHC & Mate Choice

Together these two MHC receptors serve in the recognition of foreign peptide antigens and are critical to both innate and adaptive immune responses (reviewed by Drozina *et al.*, 2005). The genes encoding the MHC receptors are highly polymorphic, providing a diverse repertoire of antigen binding capability and therefore the ability to recognize a wide array of pathogens. In addition to their critical role in both adaptive and innate immune function, these genes are now believed to play a role in mate selection (Neff & Pitcher, 2005). Intuitively, an individual possessing a heterozygous genotype should have the capacity to bind and present a variety of peptide antigens and therefore the ability to recognize a diverse range of foreign antigen. Several reports have now clearly demonstrated MHC-based mate preference in humans and mice. Olfactory studies have shown that mice can discriminate a single point mutation in MHC class I genes of their mates (Penn & Potts, 1998); however the mechanism for this mate genotype scrutiny remains controversial and unclear. Fish, which are becoming a common model to study mate choice, also demonstrate non-random fertilization (Wedekind *et al.*, 2004). Numerous studies have investigated mate selection, yet little is known of the mechanism(s) by which mate selection occurs.

1.1.5. Major Histocompatibility Receptors in Fish

Though extensively studied in mammals, MHC homologues were identified in fish more recently, the first in the common carp over 15 years ago (Hashimoto *et al.,* 1990). Since that time these genes have been identified throughout the jawed fish lineage on at least three linkage groups and for this reason should be referred to as simply MH genes (MHg; Shand & Dixon, 2001).

Despite their unusual genomic organization, fish MH receptors are believed to function like their mammalian homologues. This general acceptance is further supported through co-immunoprecipitation data (Antao *et al.*, 1999), the presence of mixed leukocyte function, immune cells, such as macrophage, as well as the identification of T cell receptors, numerous cytokines (reviewed by Stet *et al.*, 2003) and more recently the cloning of several accessory molecules (Fuller *et al.*, 2004; Fujiki *et al.*, 2003; Kales *et al.*, 2004). Unlike their mammalian counterparts, fish are subject to fluctuating seasonal body temperatures. Previous studies in mammals have revealed that cells subjected to a low, non-physiological temperature (26°C) express empty surface MHC (Ljunggren *et al.*, 1990). A subsequent study of the common carp revealed that MHC expression is maintained as low as 12°C, while fish subjected to 6°C completely abolished expression of the critical MH sub-unit, beta-$_2$-microglobulin causing a loss of MH I surface expression (Rodrigues *et al.* 1998). Expression returned following a return of the fish to warmer temperatures. Such a loss in MH I expression would greatly impair an individual's ability to recognize foreign peptides. It is interesting to note that many diseases afflicting fish demonstrate increased frequency at lower temperatures, including: winter saprolegniosis (Bly *et al,* 1992), cold-water disease (Nematollahi *et al.*, 2003) and spring viremia of carp (Ahne *et al,* 2002). These diseases are not restricted to carp and afflict most species, including the economically valuable salmonid fishes (Nematollahi *et al.*, 2003). Unfortunately, due to the lack of readily available reagents, few functional studies have investigated the regulation of fish MH expression. Our laboratory has recently developed polyclonal antibodies to several trout MH sub-units and accessory molecules. This report will review some of the recent findings using these antisera to understand the expression of MH genes, some of its accessory molecules and their role in the immune response of teleosts.

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Chapter 2

Keeping their cool:

Beta-$_2$-microglobulin gene expression is maintained in rainbow trout and Atlantic salmon kept at low temperatures.

ABSTRACT

Finfish in the wild are regularly subjected to low temperatures, which have been shown to cause a loss of Major Histocompatibility receptor expression in common carp kept at 6°. This is similar to what was seen in a mammalian cell line cultured at 26°C. Loss of expression of this critical viral recognition protein may provide one mechanism for the increased frequency of fish diseases at low temperatures. This report demonstrates that unlike carp and mammals, β_2m transcript levels in both rainbow trout and Atlantic salmon do not decrease after 10 days at temperatures as low as 2°C. Reverse transcriptase (RT) PCR indicated that transcript steady-state levels of trout β_2m were maintained in both tissues and peripheral blood leukocytes, whether freshly isolated or in primary culture. Polyclonal antibodies raised against a recombinant form of trout β_2m, demonstrated cross-reactivity to both rainbow trout and Atlantic salmon protein lysates. Use of these antibodies in western blot analyses indicated that cellular protein levels are also maintained at low temperatures in both species while qualitative epifluorescence analysis of freshly isolated peripheral blood leukocytes indicated persistent cell surface expression of trout β_2m even after 10 days at 2°C. Rainbow trout and Atlantic salmon may therefore utilize an alternative mode of immune gene regulation than the common carp and mammals allowing them to maintain viral recognition machinery at low temperatures, possibly due to selection for survival in cold climates.

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2.1. INTRODUCTION

The class I Major Histocompatibility (MH) receptor is expressed on the surface of nearly all nucleated cells and serves a critical role in self/non-self recognition in vertebrates through the presentation of endogenous peptides to circulating T lymphocytes [1]. Peptides presented by these receptors are typically generated through the proteasomal degradation of endogenous proteins derived from either self (cellular) or non-self proteins including viruses, intracellular bacteria and tumours and thereby serve as a snapshot of cellular protein processing. The class I MHC heterodimer is comprised of the membrane-spanning 45kD heavy chain, which folds to form the peptide binding groove and a 12kD, non-covalently associated beta-$_2$-microglobulin light chain, (β_2m) which has been shown to be an essential component for stable surface transport and expression [2;3]. In mammals, the genes encoding the polymorphic class I heavy chain are part of a larger group of genes which is collectively termed the Major Histocompatibility Complex, or MHC while the non-polymorphic light chain remains an unlinked single copy gene [1]. To date these genes have been identified throughout the jawed vertebrate lineage including the bony fishes, in which they are believed to function like their vertebrate homologues. Unlike their mammalian equivalents, however, these teleost homologues do not demonstrate the linkage patterns conserved among all other jawed vertebrates and are therefore referred to here as simply MH [4]. In mammals and many teleosts to date, β_2m is encoded by a single copy gene. It has been shown that rainbow trout have 3 β_2m genes expressing up to 12 allelic versions in a tetraploid manner [5].

Unlike mammals, fish with few exceptions are unable to regulate their body temperature and are therefore at the whim of seasonal environmental temperatures. In a previous study, mammalian class I MHC receptor expression was shown to be temperature dependent. When subjected to low, non-physiological temperatures (26°C), murine RMA-S cells failed to form stable class I heterodimers resulting in the surface expression of empty class I heavy chains lacking the β_2m light chain [6] and therefore the ability to present antigenic peptides [7]. A subsequent investigation using the common carp revealed that when subjected to a low, yet physiologically relevant temperature (6°C), class I heavy chain and its associated β_2m surface expression was abolished in

peripheral blood leukocytes (PBL). When these fish were returned to warmer temperatures (12°C) they regained MH class I receptor surface expression to control levels. The authors demonstrated that abolished surface expression was due to the dramatic down regulation of the β_2m transcript [8], which is considered to be the primary level of control of MHC surface expression [9]. Interestingly, carp demonstrate an increased frequency of spring viremia at these low temperatures [10]. Loss of this critical component for viral antigen recognition may provide one mechanism for such a correlation. Low temperature appears to commonly inhibit immune function in other ectotherms [11]. Among salmonid species, infectious haemotopoietic necrosis virus (IHNV) and Egtved disease correlate with temperatures, [12;13] low enough to be considered immunologically non-permissive [14]. Conservation of this phenomenon amongst all teleost fish may therefore facilitate disease susceptibility during winter months among many species. The objective of this study was to determine whether this temperature-dependent down-regulation of β_2m is conserved among the economically valuable salmonid species.

2.2. MATERIALS & METHODS

2.2.1. Fish

Two salmonid species were chosen as test models for acclimation to low physiological temperatures. Atlantic salmon (*Salmo salar*), weighing approximately 500g were kindly provided by Dr. Patrick Woo at the University of Guelph. Housed in fresh-water flow-through tanks, these fish were subjected to either 10 or 5°C for two weeks prior to sacrifice. Rainbow trout (*Oncorhynchus mykiss*), weighing approximately 500g were obtained from Rainbow Springs Trout Farm, Thamesford, Ontario and held in well-water flow-through tanks at the University of Waterloo. For low temperature challenges, trout were transferred to duplicate flow through well-water tanks with chiller by-pass inflow (Frigid Units, Inc). One week following transfer, one tank was chilled from ambient 13.5°C (+/-0.5°C) to 2.0°C (+/-0.5°C) over a 24h period, while the other was maintained with ambient well water and served as a control. Fish were maintained on a daily diet of pellet ration, then sacrificed in triplicate at days 3 & 10 by an overdose of ethyl 3 aminobenzoate methanesulphonate salt (MS222; Sigma) followed by caudal vein

exsanguination. Peripheral blood leucocytes were isolated from whole blood using histopaque 1077 (Sigma) and stored at –80°C. Tissues were extracted and flash frozen in liquid nitrogen prior to storage at –80°C. For antibody cross-reactivity testing, tissues of the common carp (*Cyprinus carpio*), were kindly provided by Dr. D. Barton and Arctic char (*Salvelinus alpinus*), by Dr. M. Powers at the University of Waterloo. For consistency, tissue samples from each species were placed in RNAlater (Ambion) at –20°C prior to extraction to reduce variability due to storage differences.

2.2.2. Northern blot analysis of Atlantic salmon tissues

Atlantic salmon were sacrificed by lethal dose of phenoxyethanol followed by caudal vein exsanguination. Whole blood was mixed with an equal volume of RPMI medium (Gibco) supplemented with 50 U/mL heparin, 150UmL penicillin, 150 mg/mL streptomycin and kept on ice during subsequent tissue extraction and 30 min. transport to the University of Waterloo. Tissues were immediately extracted, diced and placed in RNAlater for transport, then stored at –20°C. Peripheral blood leucocytes (PBL) were isolated from whole blood by centrifugation over histopaque 1077. Total RNA was isolated from washed PBL preparations using Trizol (Invitrogen) according to manufacturer's instructions. Approximately 100mgs of wet tissue was removed from RNAlater and homogenized in 4M guanidium isothiocyanate and centrifuged over a CsCl gradient. Riboprobes were generated towards previously sequenced fragments of trout elongation factor alpha (eEF1α PubMed accession # U78974) and β_2m coding sequences obtained from a trout spleen cDNA library using the following primers: β_2m sense:(5'-TGTCAATCGTTGTACTTGGG-3'; (bp 80-99), antisense:(5'-CTTCAGGTGGCGGACTCTGC-3' (bp 362-381)), eEF1α sense: (5'-GAGTGAGCGCACAGTAACAC-3'; (bp 19-38)), antisense:(5'-AAAGAGCCCTTGCCCATCTC-3'; (bp 199-218; see Eftu-1;15] Both reactions were performed using the following cycling parameters: 95°C for 5 min., 30 cycles (95°C for 45 s, 58°C for 45 s, 72°C for 45 s), 72°C for 10 min. Following sequence verification, *in vitro* transcription was performed in the presence of DIG-labelled UTP to generate DIG-labelled riboprobes according to manufacturer's instructions (Roche). Approximately 5 ug of total RNA was electrophoresed in duplicate and blotted to a MagnaCharge nylon

membrane (Osmonics Inc., Westborough, MA). Each blot was pre-hybridised with gentle shaking for 4 h at 65°C then hybridised overnight with one of the riboprobes described above in 50% formamide, 25% SSC, 0.02% SDS, 0.01% N-lauryl sarcosine in 2% blocking reagent (Roche). The blots were washed twice for 5 min in 2XSSC, 0.1% SDS at room temperature followed by one 15 min wash at 65°C in 0.5X SSC, 0.1% SDS and a final wash at 65C in 0.1X SSC, 0.1% SDS for 15 min before being equilibrated in wash buffer (maleic acid/tween 20). Bound riboprobe was detected by incubating each blot in 2% blocking reagent in 10% maleic acid using anti-DIG alkaline phosphatase Fab fragments (Roche) at 1:8000 dilution for 30 minutes. Bound antibody was detected using CDP-Star (Roche) according to manufacturer's instructions and recorded using a Fluorchem 8000 (Alpha Innotech). The band intensities for all six tissues from each fish were analyzed using spot densitometry (Alpha Innotech) for β_2m and eEF1α transcript levels. The mean signal values obtained for β_2m were compared between temperatures and the standard deviation and error were calculated for each mean value. A repeated measures ANOVA was then performed between fish (n=3), with regards to temperature and within fish, with regards to tissue type using SAS statistical software with the GLM procedure (SAS Institute Inc.).

2.2.3. RT-PCR analysis of rainbow trout tissues

Total RNA was isolated from frozen tissues and PBL pellets, as above, using Trizol. RNA yield was determined by absorbance at 260nm and diluted accordingly for cDNA synthesis using oligo dT and Advantage RT-for-PCR kit (Clontech) according to manufacturer's instructions. Subsequent semi-quantitative PCR was performed using the primers, described above for riboprobe construction at varying cycles to determine relative transcript steady-state levels. These primers cross react with all three isoforms of trout β2m. Elongation factor alpha (eEF1α) served as an internal standard for equal loading as previously described [15].

2.2.4. Cloning and development of salmonid β_2m polyclonal antibodies.

For western blot analysis, oligonucleotide sets were designed to amplify the complete 298bp mature and a 222bp N-terminally truncated coding sequence of rainbow trout β_2m. Reactions were carried out using a rainbow trout spleen cDNA library kindly provided by Dr. R. Sundick, Wayne State University using the following parameters: 95°C for 5min, 30 cycles of (95°C for 45 s, 48°C for 45 s, 72°C for 45 s), 72° for 10 min. Sense and antisense oligonucleotides were designed against published sequence (PubMed accession # L47354) to include *Bam*H1 & *Xho*I restriction endonuclease sites respectively to facilitate ligation into the expression vector, pRSETA (Invitrogen). Mature β_2m sense: (5'-CCCCGGATCCAAAGAATCTCCCCCC-3'; (bp 124-138), truncated sense: (5'-GGGGGGATCCTGTCACGTGAGTGGCTTCCACC-3'; (bp 196-217). Antisense used for both reactions:(5'- GGGGCTCGAGCCTTACATATCTGCCTC -3'; (bp 403-419). Following sequence scrutiny, the mature and truncated fusion proteins were expressed as 11.5 and 15kD inducible proteins in the expression host strain BL21(DE3) (Invitrogen), respectively (not shown). Cells were induced using 1mM IPTG for 3 h then centrifuged and lysed for protein purification under denaturing conditions using nickel affinity towards Ni-NTA agarose (Invitrogen). Pooled protein elutions were dialysed and digested using Enterokinase (Roche) to remove the fusion affinity tag according to manufacturers instructions (Roche). Each of the cleaved products was isolated and separately conjugated to KLH (Calbiochem) using glutaraldehyde (Calbiochem). Antisera were raised in two rabbits to 2 mg of each of the conjugated products using Freund's complete and then incomplete adjuvant boosts (Sigma) in 0.85% saline. Ear bleeds were regularly tested by enzyme-linked immunosorbant assay (ELISA) to analyse antibody titre. To purify rabbit sera with specific affinity for trout β_2m, the remaining mature form of purified recombinant trout β_2m was coupled to Sulfolink resin (Pierce) according to manufacturer's instructions. Peak column elutions were assessed for reactivity by western blot analysis against various tissue lysates of trout and several other species.

2.2.5. Western blot analysis of Atlantic salmon and rainbow trout tissues

Remaining Atlantic salmon tissues were dialysed out of RNAlater using three exchanges of a 1:1000 wash of PBS supplemented with 0.2mM EDTA and 0.02% sodium azide while rainbow trout tissues were simply thawed on ice prior to protein extraction. Tissues were sonicated in 500 μL of lysis buffer (1% NP-40, 150mM NaCl, 10mM Tris; pH 7.4 supplemented with 2mM phenymethylsulphonyl fluoride (PMSF; Sigma) and protease inhibitor cocktail (Sigma) per 200 mg of wet weight. Crude lysates were then cleared by centrifugation at 4°C for 10 min at 21, 000 g and the supernatant analysed by Bradford method to determine total protein concentration. Approximately 100 μg of total protein was separated by 15% acrylamide SDS-PAGE and electroblotted to nitrocellulose membrane. Total protein transfer was determined using 0.2% Ponceau S (Fisher) in 5% acetic acid and recorded using a flatbed scanner then washed and blocked for 1 h in TBS-tween containing 5% skim milk powder. Blots were probed for 1 h at room temperature using affinity-purified rabbit anti-trout β_2m antisera at a 1:150 dilution in blocking buffer. Blots were washed and then probed using goat anti-rabbit alkaline phosphatase (Sigma) at 1: 30 000 for 45 min prior to detection using NBT/BCIP and digitally recorded on a flatbed scanner. Due to differences in expression between tissues, blots were exposed for varying times to facilitate detection. For low expression tissues, (liver and muscle) blots were alternatively probed using goat anti-rabbit horseradish peroxidase at 1:2500 and detected using ECL Plus Western blot detection system (Amersham) according to manufacturer's instructions and digitally recorded using a Fluorchem 8000 imaging system (Alpha Innotech). Spot densitometry was performed using Flurochem 8000 software (AlphaInnotech) to determine β_2m band intensities relative to a conserved 42kD band detected by Ponceau staining. The mean relative ratios for each tissue type were determined from 3 fish at each temperature at both days 3 & 10. The mean relative ratios and standard deviations were plotted using Excel for Windows 2000 (Microsoft). Mean values for the two temperatures were compared by an unpaired *t*-test using GraphPad InStat (version 3.00 for Windows 95; GraphPad Software Inc.).

2.2.6. *In vitro* cell challenge

To assess expression in primary cultured cells, rainbow trout peripheral blood leukocytes were isolated, as described above, and cultured at 18°C in L-15 media (Gibco) supplemented with 15% fetal bovine serum (FBS; Gibco), 150U/mL penicillin and 150 mg/mL streptomycin for 24 h. Duplicate flasks were transferred to 2°C for 10 days, while controls remained at 18°C. Replicates were scraped and harvested at days 1, 5 & 10 from both temperatures for transcript analysis. Total RNA was isolated and utilized for RT-PCR analysis as described above.

2.2.7. Indirect epifluorescent detection of surface antigens

To detect surface expression of trout β_2m, peripheral blood leukocytes were isolated from fish after 10 days at either 2 or 13°C, as described and suspended in MEM media (Gibco) supplemented with 1% w/v bovine serum albumin (BSA; Fisher) & 0.1% w/v sodium azide. Cell suspensions were probed for 30 min at 4°C using a 1:10 dilution of purified rabbit sera while replicate controls were incubated with either: saline (no primary antisera), I-14, a monoclonal anti-trout IgM antibody or 9E1, a monoclonal anti-catfish IgM antibody. Following probing, all cell suspensions were washed three times using supplemented MEM and probed with a 1:40 dilution of either anti-rabbit or anti-mouse IgG conjugated with FITC (Sigma). Bound antibody was detected using an Axioskop 2 Plus ultraviolet microscope (Zeiss) and recorded using Northern Eclipse digital software (Empix Imaging).

2.3. RESULTS

2.3.1. β_2m transcript at low temperature

RT-PCR employing the oligonucleotide sets described above for riboprobe construction revealed no significant decrease of rainbow trout β_2m transcript levels in either freshly isolated PBL (*ex vivo*) during the 10 day challenge or in primary cultured trout PBL (*in vitro*) during a 10 day acclimation to similar temperatures (Fig. 2-1). Elongation factor 1 alpha [15] served as an internal standard of both RNA integrity and cDNA loading.

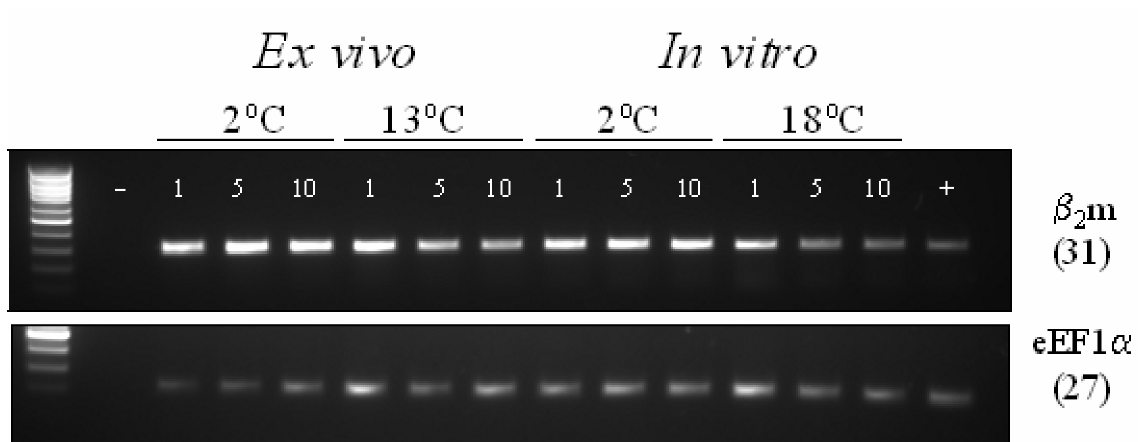

Figure 2-1. β_2m transcript steady-state levels in PBL kept at 2°C.

RT PCR analysis of β_2m transcript from freshly isolated trout PBL during acclimation to 2°C against 13°C controls (*ex vivo*) and from primary cultured PBLs during a 10 day acclimation at 2°C against 18°C controls (*in vitro*). The number of days at each temperature (1, 5 & 10) are indicated above. Elongation factor, eEF1α, served as an internal standard for both studies. Target cDNAs and PCR cycle numbers, in parentheses, are indicated on the right. A plasmid containing the full-length trout β_2m cDNA serves as a positive control template (+).

Northern blot analysis of various Atlantic salmon tissues supported the trout RT PCR data by demonstrating no statistically significant change in β_2m transcript levels in any of the tissues tested following 2 weeks at 5 & 10°C (not all blots shown; Fig. 2-2A&B). Individual two-tailed *t*-tests showed P values in excess of 0.050 between the two temperatures for each of the tissues tested. Differences between temperature groups was greatest among liver and muscle, where 5°C samples showed decreased β_2m gene expression, however neither was statistically significant. Significant differences in β_2m gene expression levels between tissues were noted, with muscle and liver being lower than intestine, spleen and gill (Fig. 2-2B).

2.3.2. Development of antibodies recognizing trout β_2m

For western blot analysis, oligonucleotide sets were used to amplify a truncated and mature sequence of trout β_2m which were sub-cloned and found to match published JB1 amino acid sequence [16], a type I trout β_2m gene [5]. Rabbit antisera generated against each of the recombinant products strongly recognized the recombinant and native forms of trout β_2m, however purified sera raised against the mature form demonstrated weaker inter-species cross-reactivity compared to those raised against the truncated protein (Fig. 2-3B &D, respectively). Antisera raised against the truncated form demonstrated variable cross-reactivity to an 11kD band in gill lysates of rainbow trout, Atlantic salmon, arctic char and the common carp (Fig. 2-3D), however subsequent tests revealed no detectable cross-reactivity to gill lysate of Atlantic cod, kindly provided by Dr. K. Gamperl, Memorial University (data not shown).

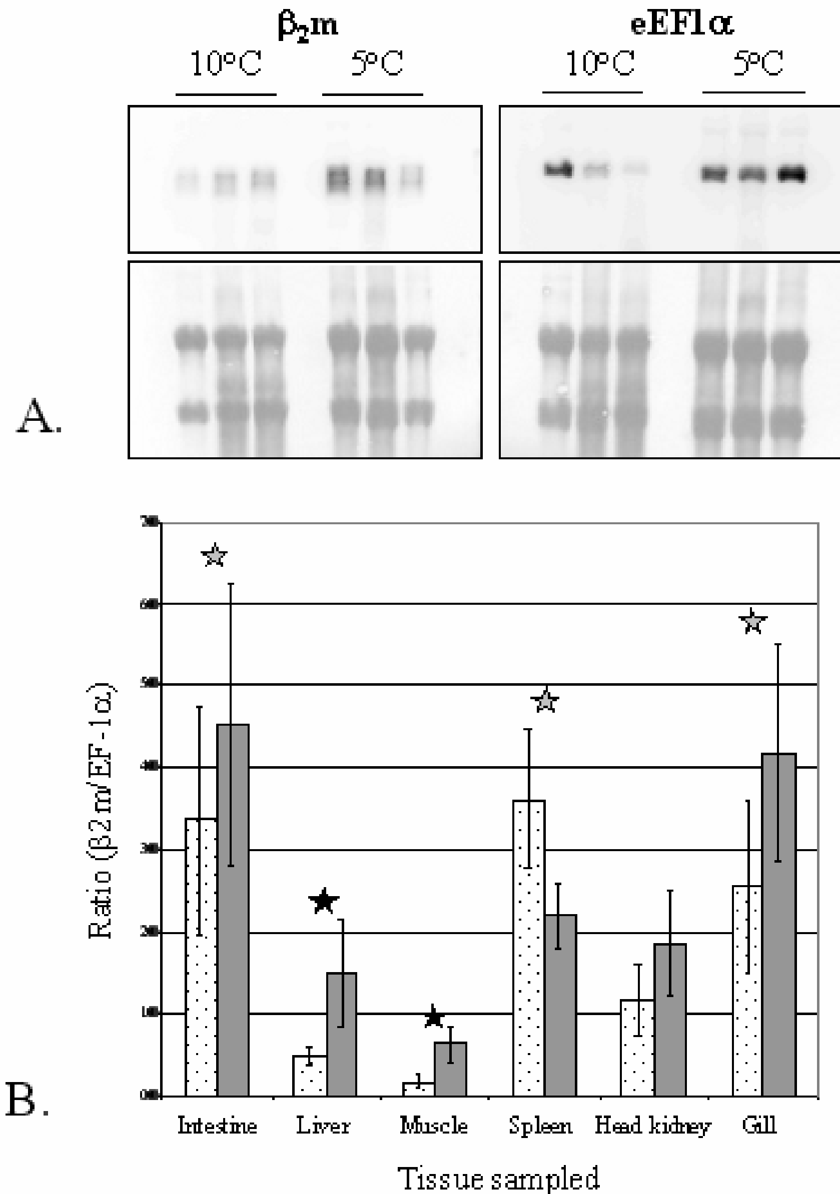

Figure 2-2. Northern blot analysis of trout β_2m transcript in Atlantic salmon kept at 5 & 10°C.

A. Northern blot analysis of Atlantic salmon PBL at 5 °C & 10°C for two weeks prior to sacrifice. Elongation factor alpha (eEF1α) serves as an internal standard, while blot stain blue below (Sigma) serves to indicate RNA transfer and integrity prior to probing. **B.** Quantification of northern blot analysis for β_2m against eEF1α ratios for all tissue types tested. Gray bars indicate ratio of β_2m *versus* eEF1α at 10°C, while spotted bars represent ratios from tissues at 5°C. Tissues showing statistically significant lower relative expression to head kidney are indicated with black stars, while those with higher relative expression are indicated with a grey star.

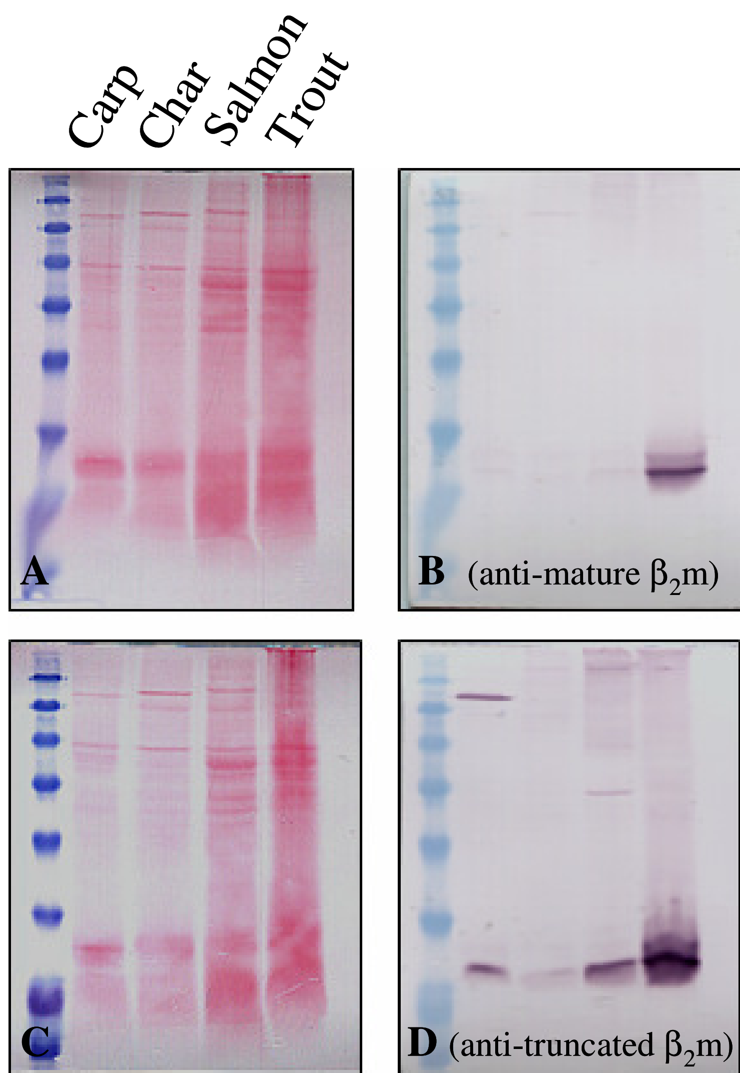

Figure 2- 3. Western blot analysis of anti-trout β_2m antiserum cross-reactivity.

Western blot analysis of anti-trout β_2m antisera cross-reactivity against gill lysates of the common carp, arctic char, Atlantic salmon and rainbow trout. Ponceau staining indicates total protein transfer in A&C. Affinity purified antisera generated towards either the mature or truncated recombinant protein demonstrates differential species cross-reactivity in B&D, respectively.

2.3.3. β_2m cellular protein expression at low temperature

Employing the antisera raised against the truncated recombinant protein, western blot analysis of Atlantic salmon tissues from three of the individuals used in northern blotting, revealed that cellular β_2m protein steady-state levels after 10 days at 5°C are maintained, as the 11kD reactive band persisted at both 5 & 10°C (Fig. 2-4). Liver lysate from one individual did demonstrate a decreased protein level and may correspond to differences seen between individuals in the northern blot analysis of these same tissues. Total protein concentration, demonstrated by Ponceau staining prior to probing served as an internal standard of protein loading and integrity indicating that such decreased liver β_2m expression is likely not due to protein degradation and may therefore truly represent a decrease in mRNA steady-state level in that individual tissue (Fig. 2-4). Increased protein levels among the 5°C muscle samples is believed to be due to increased protein loading, illustrated by the Ponceau staining below. In support of maintained salmon β_2m expression at low temperatures, western blot analysis of rainbow trout tissues and PBL lysates during a 10 day acclimation to even colder temperatures (2°C), revealed no significant loss of cellular β_2m protein steady-state levels. Banding intensity persists at levels similar to and in some tissues (liver, spleen and kidney), exceeding those in 13.5^{o}C control lysates (Fig. 2-5A). Densitometric quantification of β_2m band intensities, as a ratio to standard Ponceau staining, showed no statistically significant differences in expression levels between 2 & 13.5^{o}C for all tissues tested (Fig. 2-5 B&C) as determined by unpaired *t*-test. Therefore, like the β_2m transcript, cellular protein steady-state levels of rainbow trout are maintained at control levels after 10 days at 2°C. As demonstrated in northern blotting, differences in relative β_2m protein expression levels between tissues were noted however different detection methods employed for muscle and liver along with varied exposure times to facilitate detection between tissue types make such comparisons difficult.

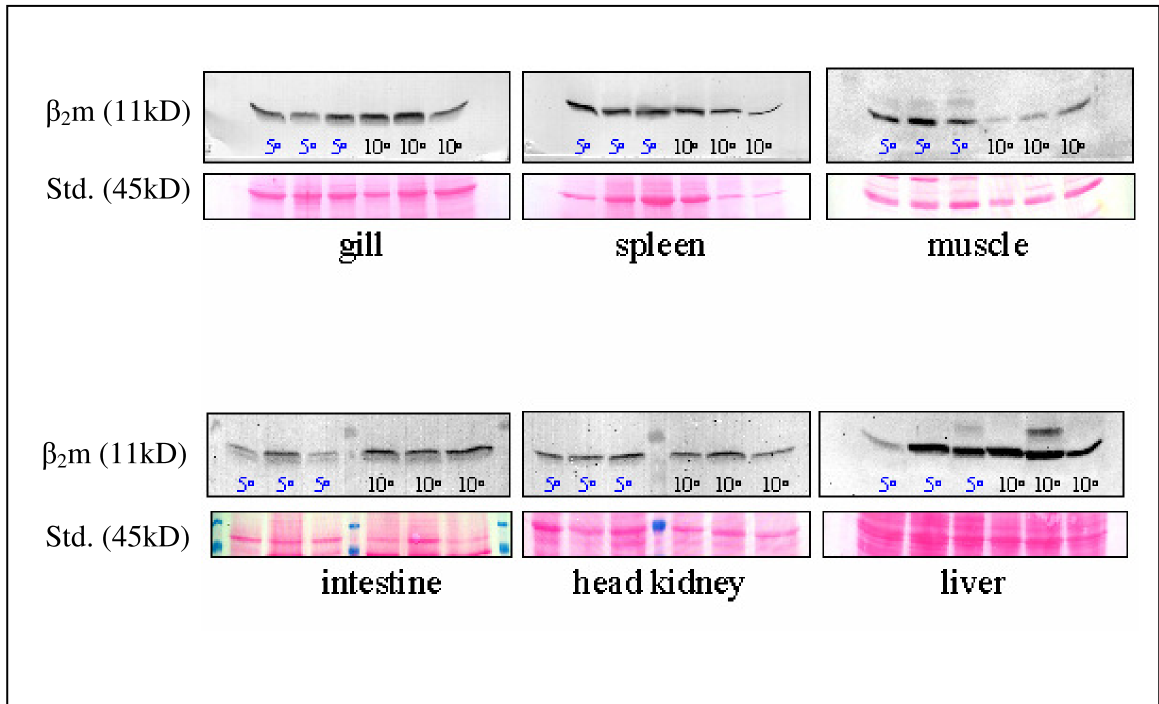

Figure 2-4. Western blot analysis of β_2m protein in Atlantic salmon kept at 5 & 10°C.

Western blot analysis of Atlantic salmon β_2m expression in various tissues after two weeks at 5 *versus* 10°C (control) using purified anti-trout truncated β_2m antisera. Ponceau staining, below, indicates total protein transfer prior to membrane probing.

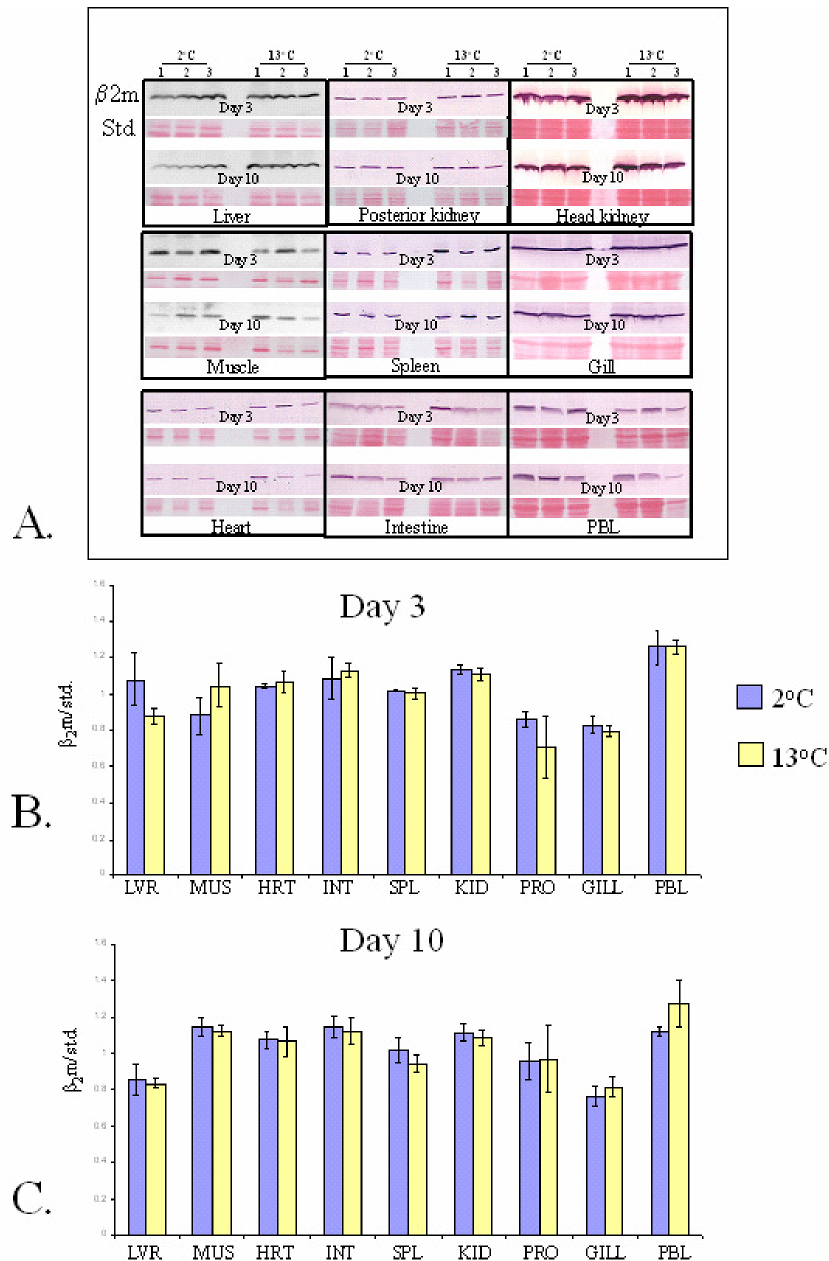

Figure 2-5. Western blot analysis of β_2m protein in tissues of rainbow trout kept at 2°C.

Western blot analysis of trout β_2m expression among various tissues during acclimation to 2°C. Tissues were collected in triplicate at days 3 & 10 from challenged and ambient control (13.5°C+/-0.5°C) fish. In panel **A**, β_2m banding at days 3 and 10 are shown with a common lysate band indicated by Ponceau staining, below, which serves as a measure of total protein transfer prior to membrane probing. Panels **B&C** show densitometric quantification of western blot analysis for β_2m bands against bands stained with Ponceau S. The ratios of intensities are shown for all tissue types tested. Light bars indicate ratio of β_2m *versus* eEF1α at control temperature, 13°C, while dark bars represent ratios at 2°C. Tissues represented include: liver, muscle, heart, intestine, spleen, kidney (posterior), pronephros (head kidney), gill and peripheral blood leukocytes.

2.3.4. Surface β_2m expression at low temperature

To address whether or not maintained cellular protein levels actually represent cell surface protein, polyclonal antibodies were employed for indirect epifluorescent detection of cell surface β_2m protein expression using FITC-conjugated secondary antibodies. Qualitative analysis indicates that cell surface β_2m protein expression is maintained after 10 days at 2°C as bound antibody was detected in PBL suspensions (Fig. 2-6). Controls indicate that neither secondary antibody alone nor catfish anti-IgM antibody react with any cell surface antigens while anti-trout IgM is reactive to only a sub-set of cells indicative of surface Ig$^+$ (sIg$^+$) lymphocytes (Fig. 2-6). Erythrocytes, (rbc) previously shown to be surface negative for these antigens in carp, [8] showed no detectable reactivity with any of the antibodies employed in this study (Fig. 2-6).

2.4. DISCUSSION

Previous studies suggest that the MH class I receptor, a critical component for the recognition of intracellular pathogens and tumours, is down-regulated at low temperatures. Rainbow trout and Atlantic salmon, both cold-water species, are frequently subjected to temperatures ranging from 15 to 0°C and are valuable species to northern fisheries and aquaculture. The goal of this study was to determine whether the β_2m gene down-regulation demonstrated in carp is conserved among these highly valued cold-water species. Unlike the common carp, steady-state transcript levels of both rainbow trout, assessed by RT PCR, and Atlantic salmon, assessed by northern blotting, appear to be maintained at 5 & even 2°C, (Fig. 2-1 & 2-2), respectively. Such data immediately imply that any temperature-dependent down-regulation of salmonid β_2m, if conserved, is not controlled at the level of transcription as demonstrated in the common carp. Given that carp only have two copies of the β_2m gene [18], it is likely that expression of these receptors is controlled through modulation of expression of this gene. Salmonids, on the other hand have 12 copies of this gene [5], and co-ordinated regulation of numerous genes may be more difficult and may explain the differences in regulatory mechanisms seen between the two species.

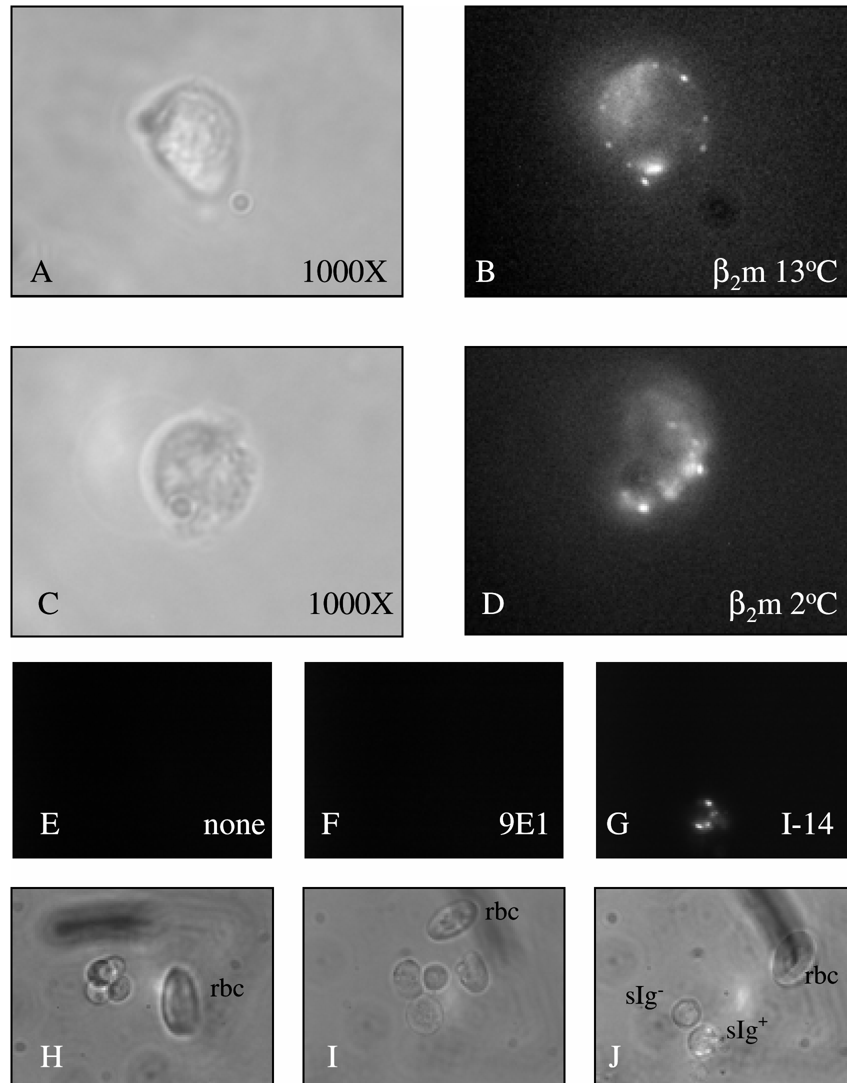

Figure 2-6. Surface β_2m expression in PBL of rainbow trout kept at 2°C & 13°C.

Indirect epifluorescent detection of trout surface β_2m after 10 days at 2 & 13°C. Peripheral blood leucocyte enhanced preparations of whole caudal blood from individuals at 2 & 13.5°C were incubated with affinity-purified rabbit anti-trout β_2m and detected using anti-rabbit IgG FITC. Bound antibody, detected through an ultraviolet (u.v.) filter, serves as a qualitative measure of surface expression. Panels A&B show bright-field and u.v. images, respectively of a PBL isolated from one individual after 10 days at control temperature. Panels C&D show the same from a PBL isolated from one individual after 10 days at 2°C. Negative controls included secondary antibody alone (anti-rabbit IgG FITC; panels E&H, respectively) and 9E1, a monoclonal anti-catfish IgM with anti-mouse IgG FITC (panels F&I). I-14, a monoclonal anti-trout IgM antibody along with anti-mouse IgG FITC serves as a positive control for surface Ig^+ cell expression (panels G&J). Erythrocytes are indicated, (rbc) in lower panels.

To address cellular protein steady-state levels among various tissues and cells, polyclonal antibodies were developed towards both a mature and truncated form of recombinant type I trout β_2m. Trout type I β_2m cDNAs differ by 5 codons at most from all known genes expressed by an individual [5;16]. Considering that only 1 of these 5 potential amino acid changes is included in the truncated clone, we believe that the subsequent polyclonal antibodies should recognize the products of all expressed β_2m genes. The antibodies developed towards these recombinant forms demonstrated varied cross-species reactivity *via* western blotting (Fig. 2-3) and allowed for detection of both the recombinant form and an approximate 11kD band from tissue lysates, corresponding to the expected size of β_2m, in both rainbow trout and Atlantic salmon (Fig. 2-3). Western blot analysis on the same tissues used for northern blot analysis revealed maintained cellular protein steady-state levels among all tissues tested (Fig. 2-4). Excluding Atlantic salmon PBL, which were exhausted in earlier northern blot analysis, all tissues demonstrated maintained translated product at such temperatures. Despite protein integrity, one individual liver lysate indicated a reduced β_2m protein level (Fig. 2-4). It is unclear whether such a loss is due to true down-regulation or simply variability among individuals as liver is known to have relatively low expression of this gene [16]. All other tissues from the same individual demonstrated control level expression at low temperature (Fig. 2-4). In rainbow trout, all tissues and PBLs clearly demonstrate maintained cellular protein steady-state levels after 10 days at 2°C (Fig. 2-5), conditions exceeding those employed upon the common carp [8]. Some tissues, such as liver, kidney and spleen demonstrated higher levels of relative expression at low temperature as compared to control. These differences are not significant and likely represent reduced rates of degradation at low temperature (Fig. 2-5). In addition, the presence of surface β_2m appears to also be maintained at 2°C (Fig. 2-6). Taken together these data indicate that, unlike carp, rainbow trout, and likely Atlantic salmon, contain persistent messenger RNA, translated product and surface expression after 10 days at temperatures previously considered immunologically non-permissive [14]. Selection pressures in colder climates may have forced rainbow trout to utilise an alternative mode of gene regulation from carp and mammals that allows for maintained viral recognition machinery at low

temperatures. It should be noted that it is unclear whether these genes are actively expressed at such temperatures or whether their products simply persist due to decreased degradation or if antigenic peptide loading and complex transport to cell surface occurs at such temperatures. Efforts are currently underway to address cell surface receptor turnover at these temperatures. Due to the differences between carp and trout transcript steady-state levels at such temperatures, we have successfully cloned the proximal promoters of rainbow trout, Atlantic salmon and carp β_2m for their use in examining the regulation of gene expression. Reporter constructs are currently being developed to further study these differences and to determine key regulatory regions that facilitate transcript temperature-independence. To this end, the apparent immunocompromised state that exists at low temperatures among these salmonid species may not be due to impaired MH class I antigen presentation machinery, as proposed in carp. Membrane fluidity, commonly known to change in response to low temperatures, has been proposed as a limiting factor to T cell activation among warmer species such as channel catfish [17]. A similar mechanism may exist among salmonids and is deserving of further attention. Considering reduced antibody response and the persistence of bacterial and fungal diseases, such as winter saprolegniosis, epizootic ulcerative syndrome and *Flavobacterium psychrophillum* among salmonids at low temperatures, class II MH temperature-dependent regulation of gene expression may serve as a limiting factor in their recognition. This possibility is currently under investigation in our laboratory.

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Chapter 3

Finding a Chaperone:

Molecular cloning and characterization of calreticulin from rainbow trout, (Oncorhynchus mykiss)

ABSTRACT

Calreticulin is a highly conserved, high capacity calcium-binding protein shared among vertebrates, invertebrates and higher plants. Its biological importance, highlighted by its highly conserved nature, is supported by its crucial physiological and immunological functions. Within the endoplasmic reticulum calreticulin serves as a calcium modulator and a lectin-like chaperone for glycoproteins, especially class I Major Histocompatibility receptors. To date, calreticulin cDNA clones have been isolated from a wide range of phyla, yet little is known about this gene in fish species, the largest and most diverse group of jawed vertebrates. This report describes the cloning of a cDNA from a rainbow trout pronephros library that encodes a deduced 419 amino acid protein, which includes a predicted 20 amino acid signal peptide and has 69% amino acid identity to both murine and human calreticulin. Like its mammalian counterparts, this cDNA endodes conserved cysteine residues believed to form a disulphide bond, a proline-rich region which includes a potential N-glycosylation site and a highly acidic C-terminal domain terminating with the ER-retrieval sequence, KDEL. Reverse transcription tissue distribution assays indicate it is ubiquitously expressed in all tissues tested with highest expression in liver while Southern blotting indicates it is a single copy gene.

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3.1. BRIEF COMMUNICATION

Calreticulin (CRT) was originally isolated from the sarcoplasmic reticulum of rabbit skeletal muscle (Ostwald & MacLennan 1974) and separately cloned in 1989 *(Fliegel et al.* 1989; Smith & Koch 1989). It has since been identified in numerous nucleated cell types (Coppolino & Dedhar 1998) and is highly conserved among vertebrates, invertebrates and higher plants (Michalak *et al.* 1999). Its ubiquity and highly conserved nature is indicative of its biological importance as a key calcium-binding protein. It is generally considered to be a resident of the endoplasmic reticulum (ER), where it serves as both a calcium modulator and chaperone to nascent glycoproteins. Through this latter function, calreticulin is believed to play a key role as a lectin-like chaperone to viral peptides (Peterson *et al.* 1995) and the class I Major Histocompatibility (MH) receptor (Sadasivan *et al.* 1996). Numerous additional functions have been postulated for calreticulin including integrin regulation (Rojiani *et al.* 1991) and as a steroid hormone receptor binding protein (Dedhar *et al.* 1994). Serum calreticulin has been shown to associate with C1q suggesting a role in both apoptotic signalling (Eggleton *et al.* 1994) as well as autoimmune dysfunctions, including systemic lupus and Sjorgren's syndrome (Rokeach *et al.* 1991). Its *in vitro* affinity for perforin and co-localization to T-cell granules also implies a role in cytoplasmic granule regulation (Fraser *et al.* 1998; 2000). To date, cDNA clones encoding this multifunctional protein have been isolated from a wide range of phyla, yet few reports provide much information on this molecule among teleosts. One clone, reported in zebrafish, suggests that it is conserved among teleosts (Rubinstein *et al.* 2000) however it has yet to be fully characterized in any fish species. In humans, this single copy gene consists of nine exons, eight introns and spans 3.6 kilobases (kb) (McCauliffe *et al.* 1992). It encodes a 400 amino acid mature protein with an additional 17 residue hydrophobic signal sequence and has a calculated molecular mass of 46 kiloDaltons (Fliegel *et al*. 1989). Since its first molecular cloning, the protein has been well characterized in mammals and consists of three domains, each with well-conserved features. The N-terminal region of mammalian CRT is highly conserved and contains three cysteines, two of which are believed to form a disulphide bond *(Højrup et al.* 2001). The P domain is a proline-rich region demonstrating high affinity Ca^{++}-

binding, which along with its chaperone capabilities, are believed to be due to the well-conserved amino acid sequence triplicate repeats: PXXIXDPDAXKPEDWDE and GXWXPPXIXNPXYX, referred to as repeats A & B, respectively, or M1 & M2 by some authors (Michalak *et al.* 1999, Li & Komatsu 2000). The remaining C-terminal region is highly acidic, demonstrates low affinity, high capacity Ca^{++}-binding and terminates with the ER-retrieval sequence, KDEL (Fliegel *et al.* 1989).

In order to clone rainbow trout CRT, oligonucleotides (sense: 5' TGACATCTGTGGCTACAGC-3' and antisense: 5'ACTCTCCCTGGTACTCTGG-3') were designed towards well-conserved regions found in a published zebrafish complete CRT cDNA (accession #AF195882) and a putative Atlantic Salmon EST, (accession #BI468042). These PCR primers were used with a rainbow trout pronephros cDNA library, which was constructed in the pcDNA3.1 cloning vector (Invitrogen, Carlsbad, Calif, USA). The resulting 423 bp amplicon was cloned into the pGEM T Easy cloning vector (Invitrogen) and sequenced using a Long-Read Tower sequencer (Visible Genetics, Toronto, Canada). This amplicon demonstrated 93% nucleotide identity to the putative Atlantic salmon EST and was subsequently labelled using the PCR DIG Probe Synthesis Kit (Roche, Basel, Switzerland) according to manufacturer's instructions. Colony hybridization, in conjunction with PCR screening, identified a tertiary positive clone of approximately 2 kb in length, which included 24 bp of 5' untranslated region (UTR) and a 1260 bp open reading frame (Fig. 3-1) demonstrating 79 and 77% nucleotide identity to a known catfish sequence (kindly provided by Dr. T. McConnell) and a published zebrafish CRT coding sequence, respectively. Additionally, a 539 bp 3'UTR containing a 12 bp poly (A) tail includes a putative non-canonical polyadenylation signal, (ATAAA), located 11 bp upstream of the poly (A) tail, similar to that found in gilthead seabream TNFα (Fig. 3-1, Garcia-Castillo *et al.* 2002). The open reading frame encodes a predicted 399 amino acid (M_r 46,000) mature peptide with an additional 20 residue hydrophobic signal sequence, determined by SignalP server (Nielsen *et al.* 1997). Amino acid sequence comparisons indicate that the N-terminal region contains two well-conserved calreticulin family signature motifs (Fig. 3-1, Li & Komatsu, 2000) as well as conserved cysteines at residues 86, 118 and 144, of which the latter are believed to form a disulphide bond (Fig. 3-2).

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                                                          CTGGAAAGGCGGGACGGTGGAAGC    24

 M  R  V  A  V  A  I  F  S  V  F  A  S  V  A  V  I  I  D  A  T  V  Y  F  K    25
ATGAGGGTCGCAGTGGCAATATTTTCAGTTTTTGCATCGGTAGCTGTCATCATCGACGCTACTGTATACTTCAAG    99

 E  Q  F  Q  D  G  D  A  W  K  S  R  W  L  V  S  K  H  K  T  D  Y  G  E  W    50
GAACAATTTCAGGATGGAGATGCATGGAAGAGCCGGTGGCTTGTATCAAAGCACAAGACTGACTATGGAGAGTGG   174

 Q  L  T  A  G  K  F  Y  G  D  A  E  A  D  K  G  L  Q  T  S  Q  D  A  R  F    75
CAACTGACTGCTGGGAAGTTTTATGGCGATGCTGAGGCCGATAAAGGCCTCCAGACCAGCCAGGATGCCCGTTTC   249

 Y  A  M  S  S  R  F  K  P  F  S  N  E  G  K  P  L  V  V  Q  F  T  V  K  H   100
TATGCTATGTCCAGCCGCTTTAAACCCTTCAGCAACGAGGGCAAGCCCCTGGTGGTCCAGTTCACTGTCAAACAC   324
         Calreticulin 1
 E  Q  K  I  D  C  G  G  G  Y  V  K  I  F  P  A  D  L  D  Q  A  A  M  H  G   125
GAGCAGAAGATCGACTGTGGCGGCGGATATGTCAAAATCTTCCCAGCAGACCTAGACCAGGCAGCTATGCACGGA   399
                              Calreticulin 2
 D  S  Q  Y  Y  I  M  F  G  P  D  I  C  G  Y  S  T  K  K  V  H  V  I  F  N   150
GACTCGCAGTACTACATCATGTTTGGGCCAGACATCTGTGGCTACAGCACCAAGAAGGTTCACGTCATCTTTAAC   474

 Y  K  G  K  N  H  L  I  K  K  E  I  K  C  K  D  D  E  L  T  H  L  Y  T  L   175
TATAAAGGCAAGAACCACCTCATCAAGAAAGAAATCAAATGCAAGGATGACGAGCTGACACACCTGTACACTCTG   549

 I  L  N  P  D  Q  T  Y  E  V  K  I  N  N  E  K  V  E  S  G  T  L  E  E  D   200
ATCCTGAACCCGGACCAGACATACGAGGTGAAGATCAACAATGAGAAGGTGGAGTCAGGCACTTTGGAGGAGGAC   624
                                    M1 repeat 1
 W  D  I  L  P  A  K  T  I  K  D  P  E  A  K  K  P  E  D  W  D  D  R  P  K   225
TGGGACATTCTGCCCGCAAAGACCATCAAGGACCCTGAGGCCAAGAAGCCAGAGGACTGGGACGACAGGCCCAAG   699
                  2                                         3
 I  D  D  P  T  D  T  K  P  E  D  W  E  K  P  E  N  I  P  D  P  D  A  K  K   250
ATTGACGATCCTACAGACACCAAGCCAGAGGACTGGGAGAAGCCTGAGAACATCCCTGACCCTGATGCTAAGAAG   774
                                             M2 repeat 1
 P  D  D  W  D  V  D  M  D  G  E  W  E  P  P  V  I  P  N  P  E  Y  Q  G  E   275
CCTGATGACTGGGATGTGGACATGGATGGAGAGTGGGAGCCTCCTGTGATCCCCAACCCGGAGTATCAGGGAGAG   849
                2                                        3
 W  N  P  K  Q  I  E  N  P  D  Y  K  G  T  W  V  H  P  E  I  D  N  P  D  Y   300
TGGAATCCAAAGCAGATTGAAAACCCCGACTACAAAGGTACCTGGGTGCATCCTGAGATTGATAACCCTGATTAC   924

 T  A  D  T  S  I  Y  K  F  D  N  I  G  V  L  G  L  D  L  W  Q  V  K  S  G   325
ACAGCTGACACCTCCATCTACAAGTTTGACAACATTGGAGTGCTGGGTCTGGACCTGTGGCAGGTGAAGTCTGGC   999

 T  I  F  D  N  F  L  I  G  D  D  V  K  E  A  E  E  F  G  N  E  T  W  G  T   350
ACCATCTTTGACAACTTCCTGATTGGTGATGATGTAAAGGAGGCGGAGGAGTTTGGAAATGAAACCTGGGGAACT  1074

 T  K  E  P  E  K  K  M  K  D  A  Q  E  E  E  E  R  K  A  R  E  E  E  E  K   375
ACAAAGGAACCAGAAAAGAAAATGAAAGATGCACAGGAGGAGGAGGAGAGGAAAGCTAGAGAAGAGGAGGAGAAG  1149

 S  K  K  D  T  A  D  D  E  G  D  D  D  D  D  E  E  D  E  S  K  E  E  E  E   400
AGCAAGAAGGACACTGCTGACGATGAGGGAGATGATGATGATGATGAGGAGGATGAGTCAAAGGAGGAGGAAGAG  1224

 D  S  P  T  E  E  G  E  E  E  T  P  K  K  D  K  D  E  L stop                 419
GACAGCCCAACGGAAGAAGGGGAAGAGGAAACTCCCAAGAAGGACAAGGACGAGTTGTAAATTAAACTGAACAGA  1299
AGATGTTTCTGTCCCGCCCTATCTTCTCTCCAATGGACTGACTGCTGTGCATCATCACATTGAGTAATACCCTAT  1374
GGACTTGCCCTTCCTGTGGCAACCTATTTTTCAGGTGTAGGGGTGGGAAAGGGGCAGGCAATGTTTGCCTTAGAG  1449
GTTCCATTTTTTTTAAACATTTTTTTTAAAATAGGTGTTTAGGGTTGAATAACTGGGTTTAAGTTTTATGTTTCT  1524
GTGAGTGATTAATTTAGGATAATTTCCCCCTTTATTCAGAATGTGGTCTGTCCTGTCCATGTGTCCTACAGTTGC  1599
TCTAGGTGGTGCTGCCACTGGCTTCCTTGCTTTTATCATCAGCTACCATCAGTTCATCCCTTCAGTGTCAAGCAA  1674
AGGATTATTCCCCACATTATTGTCATCACTCTTATCTGATATTGTCTATTTTGGGAACAAATTGGTGCATATCAG  1749
TGTAGTTGTTTGATTTATTTTATACAGTTGTATGCTGTACTGTACTATAAATTATTATAATCAAAAAAAAAAAA   1823
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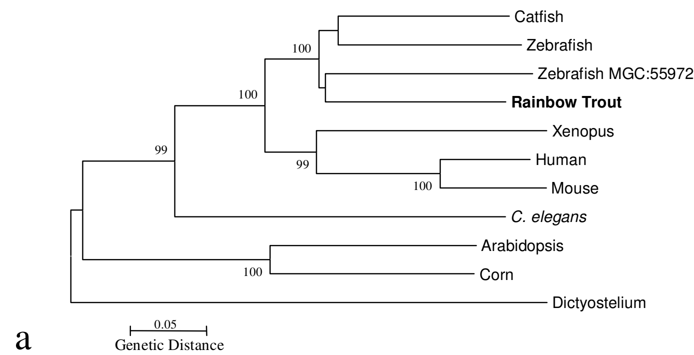
Figure 3-1. Nucleotide and deduced amino acid sequence of rainbow trout calreticulin cDNA.

Numbers on the right indicate nucleotide and amino acid positions. The predicted signal peptide is indicated with *dashed lines*. Calreticulin family signature motifs 1 & 2 are *boxed*. Triplicate repeats of M1& M2 are indicated with ***underlined bold text***. The ER retrieval motif, KDEL is indicated in ***bold text*** and a putative non-canonical polyadenylation signal is *underlined*.

Figure 3-2. Alignment of trout calreticulin with known amino acid sequences.

Alignment of derived amino acid sequence of rainbow trout cDNA with known calreticulin sequences from other organisms, including both complete and truncated piscine sequences. The predicted signal peptide is _underlined_. *Asterisks* (*) indicate gaps introduced for optimal alignment. *Dashes* (-) indicate identical sequence. Numbers on the right indicate amino acid position. An *arrowhead* (∇) indicates the position of the intron, in the genomic fragment, used to generate a genomic probe for Southern blotting. Other structural features are indicated with ***bold text*** and include: N, P & C domains, calreticulin family signature motifs 1 & 2, a potential N-glycosylation site and the ER retrieval sequence. The M1 & M2 triplicate repeats are indicated with **_bold underlined_** ***text***. Sequence data was obtained from the GenBank database with the following accession numbers: Atlantic salmon-BI468042, channel catfish (AY342298, sequence courtesy of T. McConnell - East Carolina University), zebrafish-AF195882, zebrafish MGC:55972 clone-BC046906, lamprey-AB025328, hagfish-AB025323 and mouse-BC003453.

A proline-rich region, which includes the conserved 17 residue type 1 and 14 residue type 2 triplicate repeats, M1 & M2 respectively (Fig. 3-2) suggests that teleost CRT could form the extended hairpin loop configuration previously demonstrated in rat CRT (Ellgaard *et al.* 2001). A potential N-glycosylation site at residues 345-347 is shared among some species (Michalak *et al.* 1999) but is not conserved among teleosts (Fig. 3-2). Glycosylation, which may be triggered by heat shock (Jethmalani and Henle 1998) is most common among plant calreticulins and does not appear to be a conserved feature amongst all homologues (Michalak *et al.* 1999). Like all known CRT cDNAs, the predicted C-ter minal domain of the rainbow trout clone is highly acidic, consisting of 14 and 26% aspartic and glutamic acids, respectively, and terminates with the amino acid motif KDEL, an ER-retrieval sequence (Fig. 3-2, Munro & Pelham, 1987). To further compare this clone with other known CRT sequences, a phylogenetic tree was constructed using the trout CRT cDNA sequence data along with known complete cDNAs from plants and animals (Fig. 3-3a). As expected, teleost sequences cluster together with high confidence, while mammalian and plant cDNAs each form their own distinct clusters. Interestingly, the genetic distance separating two known zebrafish sequences is greater than that of either from rainbow trout and they each cluster with a sequence from a different species rather than each other (Fig. 3-3a). Although CRT was previously reported as a single copy gene in zebrafish (Rubinstein *et al.* 2000), the second sequence (zebrafish MGC:55972) is derived from the zebrafish genome project and it is unclear whether these sequences represent alleles or separate genes. The rainbow trout sequence, reported here, may therefore be a homologue of the MGC version. Prior to this report, CRT cDNAs have only been published from zebrafish, therefore a second tree was constructed to include known piscine CRT-like sequence fragments found in GenBank corresponding to a known 660 bp Atlantic salmon EST, the shortest piscine sequence fragment (Fig. 3-3b). The trout sequence clusters closest with Atlantic salmon, while the zebrafish MGC:55972 sequence, which clustered weakly with the trout sequence in the full-length tree, now clusters even more definitively with the salmonid sequences. The zebrafish cDNA now clusters with catfish with high confidence, adding to the confusion regarding the status of the two zebrafish sequences.



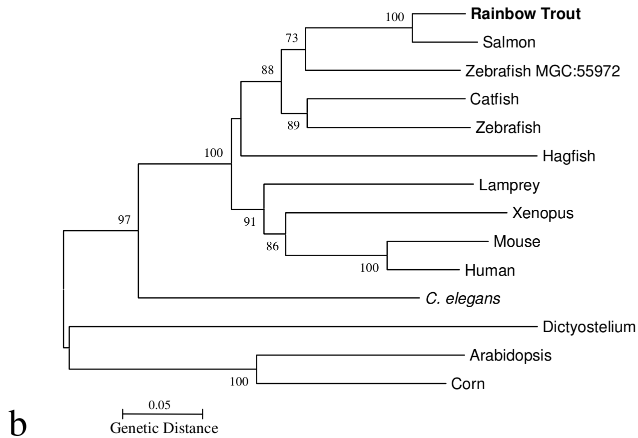


Figure 3-3. Phylogenetic analysis of known calreticulin nucleotide sequences.

a) A phylogenetic tree of calreticulin constructed using known complete cDNA sequences. The sequences were aligned using the program ClustalW (Thompson *et al.* 1994). Aligned sequences were then employed to construct a phylogenetic tree in the program MEGA2 (Kumar *et al.* 2001). The tree was constructed using neighbour-joining method (Saitou and Nei 1987) using the Jukes and Cantor correction (Jukes and Cantor 1969), pairwise deletion and 1000 bootstrap replications. *Numbers* indicate bootstrap confidence values through 1000 replications; only bootstrap values over 70 are shown. Known nucleotide coding sequences were obtained from the GenBank database, as described in the legend of figure 2. **b)** A phylogenetic tree constructed using regions of coding sequence nucleotides of known calreticulin cDNAs corresponding to a 660bp putative Atlantic salmon EST. Coding sequence nucleotides were aligned and employed, as described above.

In order to further characterize rainbow trout CRT, the primers employed above were examined for their position relative to the known mammalian CRT gene introns and appeared to span a region that should include the fifth human intron. They were therefore employed in PCR using trout genomic DNA obtained from whole blood (Sambrook *et al.* 1989) as a template. The resulting 1-kb fragment was sub-cloned and sequenced. This genomic fragment (accession number AY372527) indeed contained a 534-bp intron, including typical, eukaryotic intron splice-site signal sequences (sequence not shown) between cDNA-coding positions bp 705 and 706 (Fig. 3-2), which correspond to the position of the fifth human intron (McCauliffe *et al.* 1992). The following nested PCR primers were employed to amplify a 585-bp exon-to-intron probe: 5'-TGAGAAGGTGGAGTCAGG-3' (bp 66–84 in this fragment), 5'-ACCAGGATCACAGAAAGC-3' (bp 633–650 in this fragment). Following sequence verification, this genomic fragment was DIG-labelled as described above and employed for Southern-blot analysis. Like zebrafish (Rubinstein *et al*. 2000) and mammalian CRT genes, Southern blotting revealed that trout CRT is most likely a single-copy gene, as only one to two bands were evident in each lane of digested genomic DNA (Fig. 3-4a). This is particularly interesting, as many genes previously identified as single copy genes in mammals are often present in multiple copies in tetraploid teleosts (Schmidtke and Kandt, 1981). Such data may therefore further support the theory that many modern salmonid species are in the process of rediploidization as suggested by other trout single-copy genes including CD9 (Fujiki *et al.* 2002) and the chemokine, CK1 (Dixon *et al.* 1998; Shum *et al.* 1999). Northern blotting attempts employing the DIG-labelled cDNA probe described above failed to produce any bands in rainbow trout tissues despite the use of high-quality, undegraded total RNA. The same blot provided detection of other known transcripts, so the lack of detection was probably due to low expression of this gene. Tissue distribution assessment required more sensitive methods of detection. Therefore tissue distribution was assessed by RT-PCR employing the same RNA extracts used for northern blotting and demonstrated ubiquitous expression of CRT mRNA in all tissues and cells tested, with highest expression in liver (Fig. 3-4).

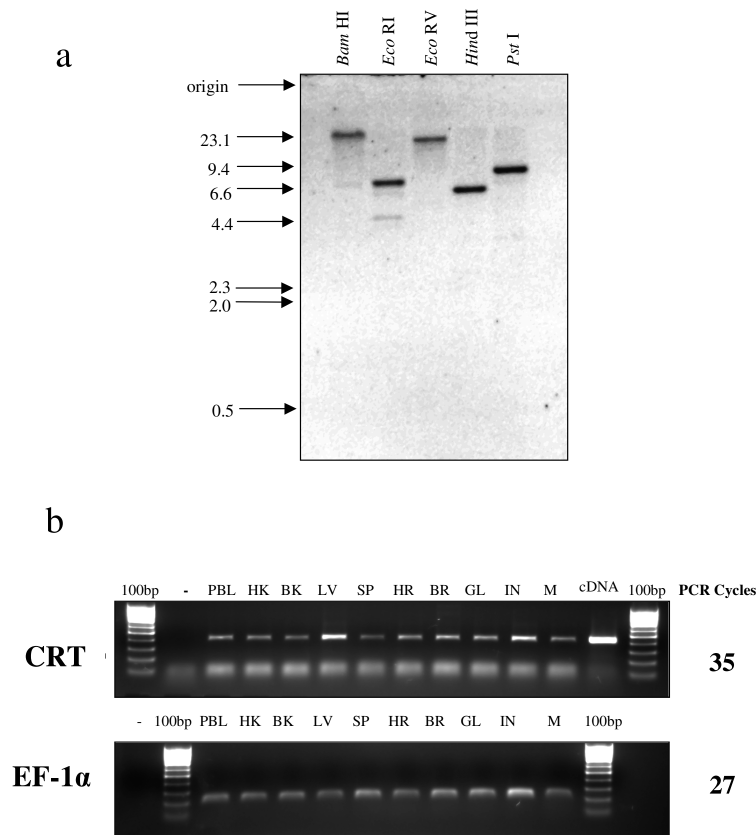


Figure 3-4. Southern blot analysis and tissue distribution assay of trout calreticulin.

a) Southern blot analysis of rainbow trout calreticulin. Ten micrograms of genomic DNA was digested using *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III and *Pst*I. Following transfer to nylon membrane, digests were hybridized with a DIG-labelled genomic probe of 585bp spanning 140 bp exon and 445 bp intron sequence. The membrane was washed twice at 65°C for 30 minutes in 0.1X SSC containing 0.1% SDS. DIG-labeled probe hybridization was detected using CDP-Star and anti-DIG antibody conjugated to alkaline phosphatase according to manufacturer's protocols (Roche). Alkaline phosphatase activity was visualized and recorded using a Fluorchem 8000 imaging system (Alpha Innotech). The origin and Lambda DNA/*Hind*III digest size markers (MBI Fermentas) are indicated in kilobase pairs on the left. **b)** Reverse transcription PCR analysis of tissue distribution of rainbow trout calreticulin expression. PCR was performed in a PTC-100 HB (MJ Research) using the following parameters: Trout Calreticulin: 96 °C for 5 minutes, 33 cycles at (96 °C for 1 minute, 54 °C for 45 seconds, 72 °C for 45 seconds) and a final elongation at 72 °C for 10 minutes. Trout elongation factor, eEF1α: 96 °C for 5 minutes, 27 cycles at (96 °C for 1 minute, 58 °C for 45 seconds, 72 °C for 45 seconds) and a final elongation at 72 °C for 10 minutes. Oligonucleotides used as PCR primers were as follows: Trout calreticulin: sense primer: 5' TGACATCTGTGGCTACAGC-3'and antisense primer: 5'ACTCTCCCTGGTACTCTGG-3'. Trout eEF1α: sense primer: 5'-GCAACCATGGGAAAGGA-3' (Genbank bp 52-68), antisense primer: 5'-TTGAAAGAGCCCTTGC-3' (Genbank bp 206-221). RNA extracted from the various tissues served as template for calreticulin amplification. Equal total RNA loading was confirmed using the internal housekeeping gene, trout elongation factor alpha, eEF1α, as an internal standard (Hansen 1997; Hansen & Strassburger 2000). Tissues and cells assayed are indicated as the following: PBL: peripheral blood leucocytes, HK: head kidney (pronephros), BK: body kidney (posterior kidney), LV: liver, SP: spleen, HR: heart, BR: brain, GL: gill, IN: intestine and M: muscle. No template controls are indicated with a *minus* (-) and plasmid cDNA served as a positive control template, indicated as cDNA.

Relatively high CRT expression in liver is a feature of mammalian CRT (Fliegel *et al.* 1989) and does not correlate with class I MH expression. This may be indicative of its many other functions, such as a steroid receptor binding protein (Dedhar *et al.* 1994). Though not unexpected, it is interesting to highlight the expression of CRT in trout peripheral blood leukocytes (PBLs). In humans, CRT has been demonstrated at the cell surface of activated peripheral T cells in association with unfolded class I MH receptors, which lack the required β-$_2$-microglobulin (β_2m; *Arosa et al.* 1999). It would be interesting to further characterize such expression among teleosts as β_2m expression is abolished following *in vivo* low-temperature acclimation in common carp PBLs (Rodrigues *et al.* 1998).

Based upon sequence comparisons and expression patterns presented above, the cDNA clone reported here appears to encode a trout homologue of CRT. *In vitro* studies are currently underway in our laboratory to investigate CRT regulation as well as sub-cellular localization and functional analyses through the development of polyclonal antibodies to recombinant trout CRT.

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Chapter 4

Calreticulin in rainbow trout:
A limited response to ER stress

ABSTRACT

Calreticulin (CRT) is a resident protein of the endoplasmic reticulum where it serves as a calcium modulator and chaperone to newly synthesized glycoproteins. In mammals, CRT is a structurally conserved 46kD protein that demonstrates anomalous migration at 60kD on SDS polyacrylamide gels and can be up-regulated by A23187 and thapsigargin due to the endoplasmic reticulum stress elements (ERSE) in the promoter region of its gene. CRT has numerous proposed functions and has been localized to the surface of PHA-stimulated T lymphocytes. CRT has been identified in mammals, plants and more recently from rainbow trout. Here, we report the cloning of the CRT proximal promoter from rainbow trout which includes elements typical of genes transcribed by RNA polymerase II including a TATA box, an Sp1 binding site, CCAAT boxes and the conservation of promoter stress elements (ERSE) demonstrated to be responsible for calcium modulation in mammals. This report demonstrates that the anomalous 60kD gel migration of mammalian CRT is conserved in trout and that CRT exists primarily as a dimer or oligomer in all tissues tested, excluding muscle and sperm in which it exists as a single polypeptide. Although it contains a potential N-glycosylation site, trout CRT is not subject to N-type glycosylation. Through the use of reverse transcriptase (RT) PCR along with western blotting, in both primary cultured leukocytes and the macrophage cell line RTS11, this report demonstrates that, unlike mammals, trout CRT is not strongly up-regulated by the calcium homeostasis antagonists, A23187 and thapsigargin, but is present on the cell surface of PHA-stimulated leukocytes. Taken together, these data suggests that CRT may have an alternative mode of regulation or function in fish.

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4.1. INTRODUCTION

4.1.1. Calreticulin in mammals

Calreticulin, (CRT) is the major calcium-binding protein of the endoplasmic reticulum (ER) and was first identified in the sarcoplasmic reticulum of rabbit skeletal muscle (Ostwald & McLennan, 1974). This protein has been isolated from numerous species of mammal, plant, a slime mold and more recently from fish (Kuraku *et al.* 1999; Rubinstein *et al.* 1994; Kales *et al.* 2004). CRT has a predicted molecular mass of 46kD with an N-terminal leader peptide and comprises three distinct structural domains. The N-terminal half of the protein consists of the N domain and P domain, so named for its unusually high abundance of proline residues. The P-domain is the site of high affinity calcium binding and contains the triplicate repeat sequences A&B (also referred to as M1 & M2), which are the hallmark of calreticulin. The remaining C-terminal half (C-domain) is highly acidic, comprising predominantly glutamic and aspartyl acids and terminates with the ER retrieval sequence, KDEL (Michalak *et al.* 1999). This highly acidic region is the site of high capacity/low affinity calcium binding and, in part, is responsible for the anomalous migration of calreticulin at 60kD in SDS-PAGE; hence its previous identification as CAB-63 (Waisman *et al.* 1986). Evidence suggests that CRT is phosphorylated by sphingosine-dependent kinases (Megadish *et al.* 1999) and through autophosphorylation at serine and threonine residues (Atreya *et al.* 1999). Other post-translational modifications include one or more potential N-glycosylation sites; however this feature shows little conservation between different species (Michalak *et al.* 1999). The proposed functions of CRT are as diverse as the organisms that express this highly conserved protein. In mammals, CRT plays a critical role in embryonic development, where mouse knock-outs are lethal by day 14 and compromised expression of CRT has been shown to disrupt normal cardiac development and function. In mouse fibroblasts, CRT has been shown to modulate gene expression and cell adhesiveness through its interaction with integrins (Michalak *et al.* 1996; Opas *et al.* 1996). Though considered an ER-resident protein with its KDEL retention sequence, this protein has been localized to the surface of activated T lymphocytes and demonstrates perforin affinity suggesting a role in the regulation of T cell killing (Arosa *et al.* 1999). CRT has also been found in the

acrosome of spermatids where it may play a role in spermatogenesis and motility, as mouse sperm is immobilized following exposure to CRT antisera (Nakamura *et al.* 1992; Nakamura *et al.* 1993). Within the ER, CRT is generally considered to perform two main functions: as a calcium modulator and chaperone to newly synthesized glycoproteins, especially those destined for secretion (DiJeso *et al.* 2003), including the class I Major Histocompatibility Complex (MHC) receptor and most other immune proteins. As a calcium modulator, CRT plays a key role in Ca^{++}-mediated apoptosis (Demaurex & Distelhorst, 1993) and demonstrates cycloheximide-dependent up-regulation in response to the calcium homeostasis antagonists (CHA), thapsigargin and calcium ionophore (A23187; Waser *et al.* 1997; Llewellyn *et al.* 1996). Calcium ionophore and thapsigargin, a known inhibitor of Ca^{++}-ATPases, lead to a depletion of ER Ca^{++} ions. The five to seven-fold increase in CRT expression, demonstrated following thapsigargin treatment, is believed to compensate for Ca^{++} homeostasis disruption through its high capacity Ca^{++} binding (Waser *et al.* 1997). Some data also suggests that CRT up-regulation may occur in response to other stressors including heat shock (Conway *et al.* 1995; Nguyen *et al.* 1996) and viral infection (Zhu, 1996). Reporter knock-out assays have demonstrated that CRT gene expression is regulated through conserved endoplasmic reticulum stress elements (ERSE) within its promoter region (Kokame *et al.* 2001). ERSE sequences, possessing the consensus motif CCAAT-N_9-CCACG, are necessary for induction of calreticulin and other known ER proteins, including: Herp, GRP78 (BiP) and GRP94 through the unfolded protein response (UPR; Kokame *et al.* 2001; Yoshida *et al.* 2003). The UPR is believed to function through binding of the transcription factor, ATF6 to ERSE promoter elements resulting in enhanced endogenous CRT expression (Yoshida *et al.* 2003). Promoter deletion assays have revealed that the response to CHA is controlled by these same promoter regions (Waser *et al.* 1997) indicating that ERSE sequences are critical for the regulation of calreticulin gene expression in relation to its two main functions. Through its many functions, CRT plays an intimate role in immune competence. As a primary chaperone to glycoproteins such as the MHC receptor, CRT is an indirect determinant of allograft, tumour and viral recognition. CRT and its capacity to regulate perforin, though still unclear, indicates a role in cytotoxic T cell function, while

evidence suggesting that CRT serves as a C1q co-receptor implicates it in complement function (Kishore *et al.* 1997; Eggleton *et al.* 1994).

Due to its affinity for glycoproteins, CRT has also been shown to serve as a danger-signaling molecule by eliciting a cytotoxic response to viral and tumour peptides, a role shared with some heat shock proteins (Berwin & Nicchitta, 2000). Taken together, this evidence suggests that CRT plays a pivotal role in regulating not only one of the most important secondary intracellular messengers, Ca^{++}, but also innate and adaptive immune function.

4.1.2. Calreticulin in fish

CRT was first identified in fish through its differential expression pattern in zebrafish *cyclops* mutants. The *cyclops* mutation produces embryos lacking a spinal cord floor plate and ventral brain, which normally separates the eyes, resulting in cyclopia (Hatta *et al.* 1994). Through differential gene expression screening of wild-type and mutant cDNA pools, zebrafish mutants showed an altered expression profile of what was later identified as the homologue of calreticulin (Rubinstein *et al.* 2000). Since that time, CRT has been fully characterized in only one fish species, rainbow trout, where it was shown to be expressed in all tissues tested and is likely a single copy gene exhibiting 69% amino acid identity to both human and mouse CRT (Kales *et al.* 2004). Phylogenetic analysis has revealed that one independently isolated zebrafish allele was more similar to trout CRT than to other alleles of the same species suggesting the possibility that zebrafish, and perhaps some tetraploid fishes, may contain two divergent copies of this gene (Kales *et al.* 2004). Considering the important role this protein plays in embryonic development, calcium homeostasis and immune function, much still remains unknown about this protein in fish as well as its many implied functions in mammals. Here, we report the cloning of a 457 bp region of the CRT proximal promoter from rainbow trout. Also through the use of reverse transcriptase (RT) PCR and the development of trout CRT-specific antibodies, this report investigates the expression patterns of trout CRT in response to CHA. Finally, trout CRT is examined for conserved features demonstrated in mammalian models.

4.2. MATERIALS AND METHODS

4.2.1. Cloning of Trout Calreticulin Promoter Sequence

Genomic DNA (gDNA) was isolated from the whole blood of one individual rainbow trout according as previously described (Sambrook *et al.* 1989). Genome Walker library templates were generated using Universal Genome Walker Kit adapters, according to manufacturer's instructions by digesting trout whole blood gDNA using 5 blunt-end endonucleases followed by ligation using kit adapters provided (Clontech Laboratories, Mountain View, USA). Primary PCR reactions were performed against pooled adapter templates using oligonucleotides provided along with the following gene specific oligonucleotide designed against published rainbow trout cDNA sequence (Genbank accession #AY372389: DAW65rev antisense: 5'-TTCCATGCATCTCCATC-3' (bp#405-419)). Nested PCR was then performed on the *Pma*C I digest template according to manufacturer's instructions using a second oligonucleotide: RTCRTrev2 antisense: 5'-ATGATGTAGTACTGC-3' (bp#111-128)). Nested PCR amplicons were separated by electrophoresis and extracted from 1% agarose using Ultrafree DA columns (Millipore, Billerica, USA). Fragments were ligated into the pGEM T easy vector prior to sequencing. Dye-terminating reactions were performed using vector-specific primers and analyzed at the University of Waterloo using a long read tower sequencer according to manufacturer's instructions (Visible Genetics). The amplified promoter region of rainbow trout CRT was analyzed for known promoter elements using Signal scan (Prestridge, 1991) and through comparison with published mammalian CRT promoter sequence.

4.2.2. Production of recombinant trout CRT

Oligonucleotides were designed using published cDNA sequences to amplify the complete coding region of rainbow trout CRT (AY372379; bp# 25-1284). 5' GC clamps preceding *Bam*HI and *Xho* I restriction sites were included in the sense and antisense primers, respectively to facilitate enzymatic digestion and subsequent ligation into the prokaryotic expression vector, pRSET A (Invitrogen). PCR primers and cycling parameters were performed as follows using the cDNA originally isolated from a rainbow trout spleen cDNA (Kales *et al.* 2004) as a template: RTCRTBamsense (5'-GCGCGGATCCACTGTATACTTCAAGGAAC-3'), RTCRTXhoantisense (5'-GCGCCTCGAGTTACAACTCGT-3'), 95°C for 5 min., 30 cycles (95°C for 45s, 44°C

for 45s, 72°C for 1m 30s), 72°C for 10 min. The resulting 1,260bp amplicon was ligated into pGEM T easy (Promega, Madison, USA) and sequenced, as described above. Following sequence scrutiny, the amplicon was excised using a *Bam*HI and *Xho*I (MBI Fermentas) double-digest at 37°C overnight (Invitrogen) and purified by 1% agarose gel electrophoresis extraction, as described above. Similarly, the pRSET A expression vector was prepared for ligation using *Bam*HI and *Xho*I digestion followed by a 30 min incubation with calf intestinal alkaline phosphatase according to manufacturer's instruction (CIAP; Promega). The purified CRT coding sequence was ligated into the pRSET A backbone by overnight incubation with T4 ligase (Promega) at 4°C. Following sequence verification, the pRSET A-CRT fusion vector was transformed into *E. coli* BL21 SI competent cells (Invitrogen). These cells were made competent by calcium chloride method (Sambrook *et al.* 1989) and contain a salt-inducible T7 RNA polymerase vector which regulates the up-stream promoter of the pRSET A vector. Transformed cultures were screened from a modified Luria-Bertoni (LB) broth lacking the addition of NaCl and containing 100 µg/mL ampicillin at 37°C with shaking to an OD600=0.5 prior to induction using 250 mM NaCl. Cultures were pelleted after 3h and lyzed using denaturing conditions (8M urea) according to manufacturer's instructions (Invitrogen). Recombinant trout CRT, which was a fusion peptide containing an N-terminal 6XHis tag and AntiXpress epitope, was purified using Ni-NTA agarose according to manufacturer's instructions (Invitrogen) and verified by western blotting using AntiXpress antibody (Invitrogen).

4.2.3. Development of Trout Calreticulin Antisera

Pooled elutions of purified recombinant trout CRT were dialyzed out of denaturing buffer using a gradual four-step-wise decrease in urea concentration into 1X PBS. A total of 3 mg of recombinant protein was injected intramuscularly (*i.m*) into 2 New Zealand white rabbits emulsified 1:1 in Freund's complete adjuvant (FCA) followed by 3 subsequent boosts at 3 week intervals using Freund's incomplete adjuvant (FIA). Ear bleeds were regularly performed to analyze rabbit antibody titre prior to exsanguination. Subsequent polyclonal antibodies were purified from the rabbit serum using Sulfolink resin charged with 3 mg of purified recombinant trout CRT according to manufacturer's instructions (Pierce).

4.2.4. Rainbow trout tissue & cell preparations for *in vitro* challenge

For tissue analysis, rainbow trout weighing approximately 500 g, were euthanized using a lethal dose of MS-222 (Sigma) followed by caudal vein exsanquination using a heparinized needle. Tissues were extracted and flash frozen in dry ice prior to storage at –80°C. For peripheral blood leukocyte (PBL) enriched blood preparations, caudal blood was centrifuged at 1000 g for 10 minutes. The buffy coat was transferred to a total volume of 25 mL in L-15 medium containing 50 U/mL heparin and split into 3 equal portions that were each layered above 3 mL of Histopaque 1077 (Sigma) and centrifuged at 1000g for 30 min. The PBL layer was collected and washed using 1X PBS and transferred to treated 6 well culture plates (Nunc) containing L-15 medium supplemented with 10% fetal bovine serum (FBS; Gibco), 150 U/mL penicillin and 150 mg/mL streptomycin at 2.0 X 10^{7} cells/mL and allowed to attach overnight. The rainbow trout macrophage cell line, RTS11 (Ganassin & Bols, 1998) was employed to assess the effects of CHA in a more stable and homogenous cell population. Prior to drug treatments, cells, at concentrations of 2.0 x10^{6} cells/plate, were allowed to attach overnight in 25 cm^{2} treated cell culture dishes (Nunc) with L-15 medium supplemented with 150 U/mL penicillin and 150 mg/mL streptomycin and 15% fetal bovine serum (FBS). PBL and RTS11 cultures were treated with 100 nM Thapsigargin, 7 ug/mL A23187 (Sigma) or equivalent volumes of DMSO solvent (not exceeding 0.5% final concentration) as a vehicle control. To mimic dsRNA, typically present during viral challenges, treatments also included 50 ug/mL polyinosinic-polycytidylic acid (poly I:C; Sigma) or an equivalent volume of filtered water as a vehicle control. Cultures were observed and harvested at several time points throughout 24 h challenges.

4.2.5. RT PCR analysis of Trout Calreticulin Transcript

Total RNA was isolated from frozen tissues and cell pellets using Trizol (Invitrogen) according to manufacturer's instructions. RNA yield was determined by absorbance at 260 nm and diluted accordingly for cDNA synthesis using oligo dT and Advantage RT-for-PCR kit (Clontech) according to manufacturer's instructions. Subsequent PCR was performed using the following primers designed to amplify a 453bp, (bp#429-850), coding region of published sequence, (PubMed accession number: AY372379), at varying cycles to determine relative transcript steady-state levels: RTCRTF1 sense (5'-

AGACATCTGTGGCTACAGC-3') & RTCRTrev1 antisense (5'-ACTCTCCCTGATACTCCGG-3'). Elongation factor alpha (eEF1α) served as an internal standard for equal loading as previously described (Thompson, 1988) using the following primer set: eEF1α sense: (5'-GAGTGAGCGCACAGTAACAC-3'; bp# 19-38)), antisense:(5'-AAAGAGCCCTTGCCCATCTC-3'; (bp# 199-218)) (see Eftu-1 in Hansen & Strassburger, 2000; PubMed accession number: U78974). PCR reactions were performed for both cDNA targets using the following cycling parameters: 95°C for 5 min., # cycles (95°C for 45 s, 54°C for CRT or 58°C for eEF1α for 45 s, 72°C for 45 s), 72°C for 10 min. Amplicons were electrophoresed over 2% agarose containing ethidium bromide and recorded using a Fluorchem 8000 gel imager (AlphaInnotech).

4.2.6. Western Blot Analysis

Approximately 200 mg of wet tissue or cell pellet was thawed and sonicated in 500 uL of lysis buffer (1% NP-40, 150mM NaCl, 10mM Tris; pH 7.4 supplemented with 2mM phenymethylsulphonyl fluoride (PMSF; Sigma) and protease inhibitor cocktail (Sigma)). Crude lysates were then cleared by centrifugation at 4°C for 10 min at 21,000 g and the supernatant analysed by Bradford method to determine and equalize total protein concentration among samples. Approximately 100 μg of total protein was separated by 15% SDS-PAGE and electroblotted to nitrocellulose membrane. Total protein transfer was determined using 0.2% Ponceau S (Fisher) in 5% acetic acid and recorded using a flatbed scanner then washed and blocked for 1 h in TBS-Tween containing 5% skim milk powder. Blots were probed for 1 h at room temperature using affinity-purified rabbit anti-trout CRT antisera at a 1:100 dilution in blocking buffer. Blots were washed three times in TBS-Tween for 5 min each, then probed using goat anti-rabbit alkaline phosphatase (Sigma) at 1: 30 000 for 45 min prior to detection using NBT/BCIP and digitally recorded on a flatbed scanner. Equal loading was determined by relative band detection following Ponceau staining.

4.2.7. De-glycosylation

For *in vitro* de-glycosylation, trout gill lysate containing approximately 500 μgs of total protein was concentrated using a 3 000 MWCO spin column (Millipore) according to manufacturer's instructions. Lysate proteins were resuspended in 200 μL of sterile

distilled water containing 0.5% SDS, 0.1% β-mercaptoethanol and boiled for 10 minutes. The cooled lysate was treated overnight at 37°C with 2500 U of Endo H (New England BioLabs) in a 50 mM sodium citrate buffer; pH 5.5 followed by an additional overnight incubation at 37°C following the addition of 20 U N-glycosidase F (Roche) in 50 mM sodium phosphate; pH 7.5 containing 1% NP-40. A mock treatment (-), containing equivalent volumes of purified water in place of enzymes, served as reaction control. Reaction volumes equivalent to 50 μgs of original total protein were separated on 15% SDS-PAGE and blotted onto nitrocellulose membrane for subsequent probing using rabbit anti-trout CRT antisera, as described. To verify sufficient de-glycosylation, blots were also probed using anti-trout MH I heavy chain antisera, a protein known to contain a functional N-glycosylation site (Kales, 2006).

4.2.8. Indirect epifluorescent detection of surface CRT

To detect surface expression of trout CRT, peripheral blood leukocytes were isolated as described and transferred to culture treated 25 mm plates (Nunc) containing L-15 medium supplemented with 10% fetal bovine serum (FBS; Gibco), 150 U/mL penicillin and 150 mg/mL streptomycin at 2.0 X 10^7 cells/mL and allowed to attach overnight. Cells were then treated with 5 μg/ml phytohemagglutinin, (PHA) while a replicate plate was supplemented with an equivalent volume of PBS to serve as a vehicle control. Cells were collected after 5 days, washed and suspended in L-15 media (Gibco) supplemented with 1% w/v bovine serum albumin (BSA; Fisher) & 0.1% w/v sodium azide. Cell suspensions were probed for 30 min at 4°C using a 1:10 dilution of purified rabbit anti-trout CRT while replicate controls were incubated with either: saline (no primary antisera), pre-immunization sera or affinity-purified rabbit anti-trout β_2m (Kales *et al.* 2006). Following probing, all cell suspensions were washed three times using supplemented media and probed with a 1:40 dilution of either anti-rabbit or anti-mouse IgG conjugated with FITC (Sigma). Bound antibody was detected using an Axioskop 2 Plus ultraviolet microscope (Zeiss) and recorded using Northern Eclipse digital software (Empix Imaging).

4.3. RESULTS

4.3.1. Protein Expression & Antibody development

Salt induced *E. coli* BL21 SI cells, transformed with the pRSET A-trout CRT fusion vector, expressed a protein with strong reactivity to the AntiXpress antibody (Invitrogen) and relative molecular mass of approximately ~64 kD (not shown). In western blots, affinity-purified polyclonal antisera, raised in rabbits towards this purified recombinant product, demonstrated reactivity to multiple polypeptides having molecular mass of approximately 60 kD in all trout cell and tissue lysates tested, excluding muscle and sperm lysate, which contained only a single reactive band of smaller relative molecular mass (Fig. 4-1A&B). Seminal fluid (SF) and blood plasma (PL) lacked any detectable CRT (Fig. 4-1A) while relative protein expression levels appeared highest in liver, supporting previous transcript expression data (Kales *et al.* 2004). Spleen and intestine demonstrated the most abundant polypeptides, as reactive bands of several different sizes were apparent (Fig. 4-1A). For comparison, trout β_2m, shown below, was highest in spleen and gill and lowest in muscle, as previously reported (Shum *et al.* 1996) while undetectable in red blood cells, yet present in brain. Like trout β_2m, which is not subject to N-type glycosylation, the CRT reactive bands in gill lysate appeared unaffected following *in vitro* de-glycosylation (Fig. 4-2) suggesting that the multiple bands observed are not due to differential N-type glycosylation and that trout gill CRT is not subject to N-type glycosylation.

4.3.2. Cloning of rainbow trout calreticulin promoter region

Genome walking of rainbow trout genomic DNA templates, isolated from whole blood, generated a 559 bp genomic fragment which included 102 bp of 5' coding sequence matching previously published rainbow trout CRT as well as 457 bp of 5' untranslated (UTR) sequence (Fig. 4-3). This region included 431 bp of novel upstream sequence (Fig. 4-3). which contained several putative regulatory sequences typical of genes transcribed by RNA polymerase II, including a GC rich region, a TATA box at position -38, a YY1 site at position -57 and an Sp1 binding site at position +9 (Fig. 4-3). The trout CRT promoter also includes 2 CCAAT boxes, each containing ERSE-like sequences beginning at positions -117 & -131 and having the consensus sequence CCAAT-N_9-CCACG.

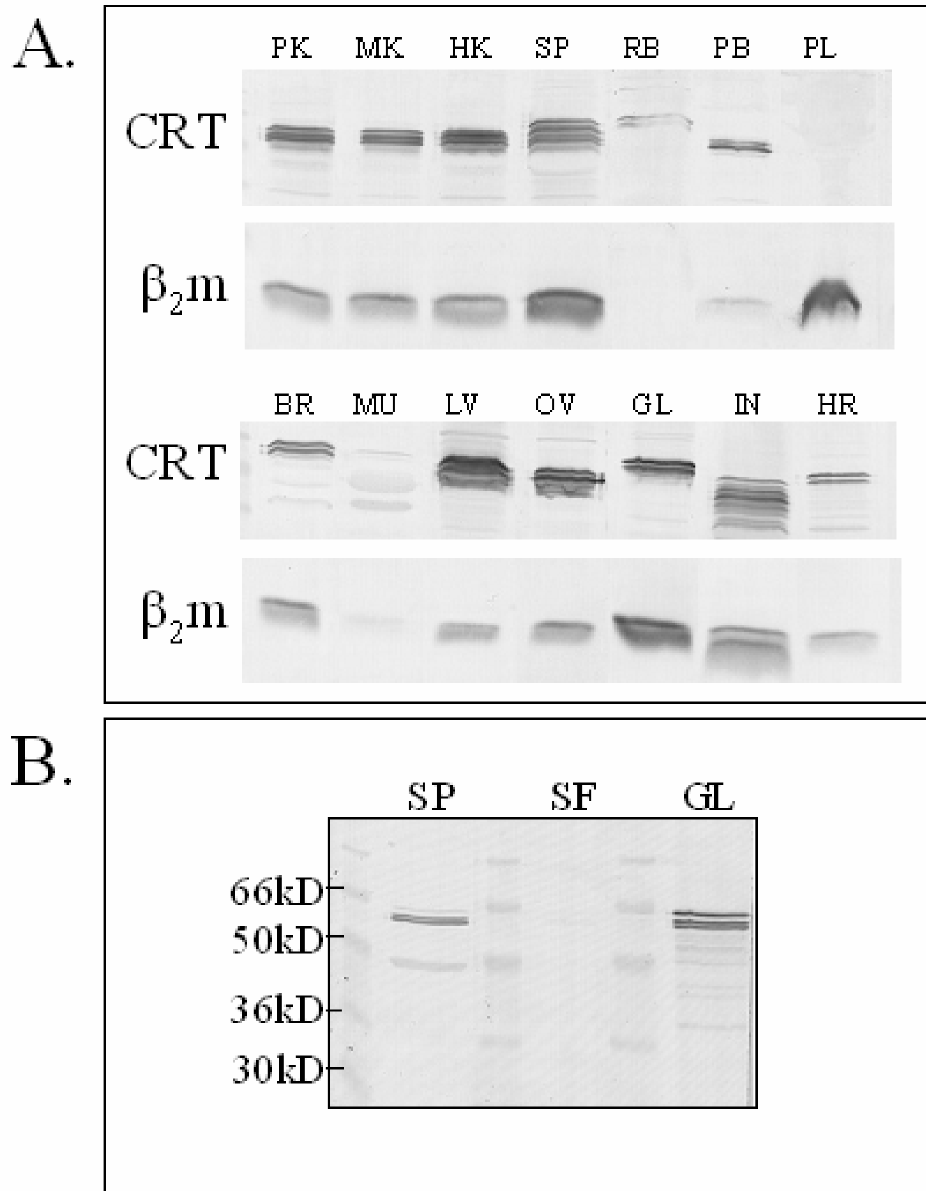

Figure 4-1 Tissue distribution of trout calreticulin protein expression.

(A) Western blot analysis of CRT protein tissue distribution in one individual trout. Lysates were equalized and each represent aproximately 100µgs of total protein, based upon Bradford analysis. Tissues and cell lysates are indicated above as brain (BR), ovary (OV), posterior-kidney (PK), Gill (GL), liver (LV), muscle (MU), mid-kidney (MK), spleen (SP), head kidney (HK), heart (HR), intestine (IN), red blood cells (RB), white blood cells (WB) & blood plasma (BP). (**B**) Western blot analysis demonstrating differential CRT protein expression in sperm cells, (SP), seminal fluid (SF) and gill (GL) in a male individual.

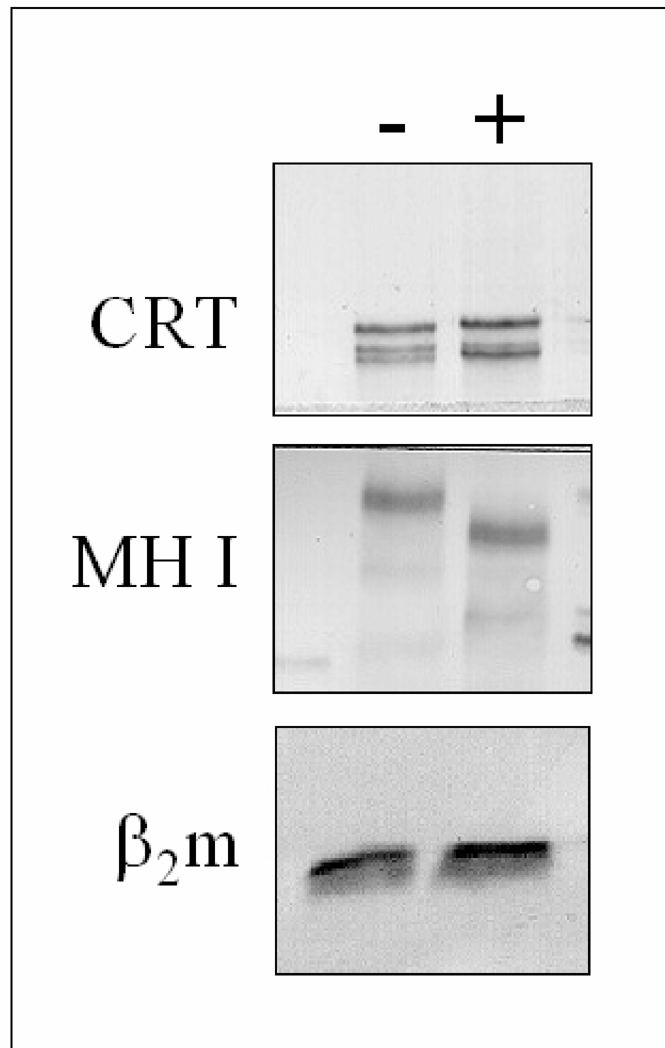

Figure 4-2. Western blot analyses of trout gill lysate following *in vitro* deglycosylation.

Mock reactions containing no enzyme are indicated above with a minus sign (-). Proteins tested include trout CRT, trout MH I heavy chain, known to contain one N-glycosylation site and trout β2m, known not to contain any glycosylation.

```
        GC box
CGACGGCCCGGGCTGGTGTGTCCTACTTATATCGCTACACTCATGACAACCTAAAC  -378

ATTACAAAACTTCTATTCGATCAAATAAACTTCACGTAGCAAATAAATCATTCGTT  -322

TTTTGTTGACCAAATTCGACACTCGCTCATTGACCTCCATACAAAAACTCCTTGCT  -266

TGGTGTTCGAAAAAACGTAACCTGCTGGATGGAGGGAGACAGATTTTCCGTCGAGT  -210

TGGGCCTCTCTCTTTGACTCTTCCTCTCTGTGTGTAATCCGAGCACTTCCGAAAAA  -154
            ERSE                                ERSE
TAACTCGTGGTCTCATTTCATTGGACAGGATCACATCCAATGAGCTGCGGCCATCT  -98
     <------------------               ----------------->
                                                YY1
GTGAAGGATACGGAGTTAGAAATGATCATATAACCTGTTTCCTTATTCTTTGAGCG  -42
   TATA box                                   ↓        Sp1
TTATATTACTGGGTTTCAAGGAGCTAAACGACACAGTTAACCTGGAAAGGCGGGAC  +15
           M  R  V  A  V  A  I  F  S  V  F  A  S  V  A
GGTGGAAGCATGAGGGTCGCAGTGGCAATATTTTCAGTTTTTGCATCGGTAGCTGT  +71
V  I  I  D  A  T  V  Y  F  K  E  Q  F  Q  D  G  D  A  W
CATCATCGACGCTACTGTATACTTCAAGGAACAATTTCAGGATGGAGATGCATGG   +127
```

Figure 4-3. Nucleotide sequence of putative trout CRT proximal promoter.

Position +1 of the nucleotide sequence is defined as the most upstream of known transcribed nucleotide and is indicated with an arrow. Typical RNA polymerase II binding sites are indicated above including: a GC box, TATA box, an Sp1 binding site as well as a YY1 site and two ERSE sequences, one in forward and the other in reverse orientation. Derived amino acid sequence is indicated above the CRT leader peptide and coding sequence.

This matches ERSE sequences previously described in human CRT (Yoshida *et al.* 2003), which have been found to be necessary and sufficient for UPR and CHA induction of other known ER-resident chaperones.

4.3.3. *In vitro* challenge & trout calreticulin steady state levels in primary cultured cells

Unlike mammalian CRT, trout CRT failed to demonstrate strong up-regulation of transcript steady-state levels following *in vitro* CHA treatment. Calcium ionophore and thapsigargin, both of which have been shown to cause a five-fold increase in mammalian CRT expression (Waser *et al.* 1997), failed to induce a similar response in CRT transcript in primary cultured trout PBL (Fig. 4A). Other *in vitro* challenges, including heat shock (28°C for 24 h), LPS (100 µg/mL for 24 h), PMA (100 µg/mL for 24 h) and Gliotoxin (10 ng/mL for 24h) also failed to cause any significant changes in trout CRT expression at the transcript level (not shown). Due to the presence of ERSE-like promoter elements, CHA treatment was employed to assess expression at the protein level using western blotting. Like the transcript analysis, overall protein steady-state levels appeared to not be up-regulated after 24 h CHA treatment of trout PBL (Fig. 4-4B), however cytological effects were clearly evident during the course of the treatment as cell population sub-structure changed dramatically with respect to controls (Fig. 4-4C). PBL-enriched blood treated with thapsigargin in primary culture demonstrated a selective decrease of leukocytes (Fig. 4-4C; Thaps.), while A23187 appeared to cause a loss of erythrocytes and selection for larger white blood cells (lymphocytes) *via* apoptosis of the smaller cells, as phase-bright blebbing was apparent (Fig. 4-4C; A23187). DMSO vehicle control plates, along with the no treatment control retained their original mixed population characteristics (Fig. 4-4C; DMSO & NONE). PolyI:C, a synthetic double-stranded RNA, used to mimic viral infection also failed to elicit any significant transcript or cytological change.

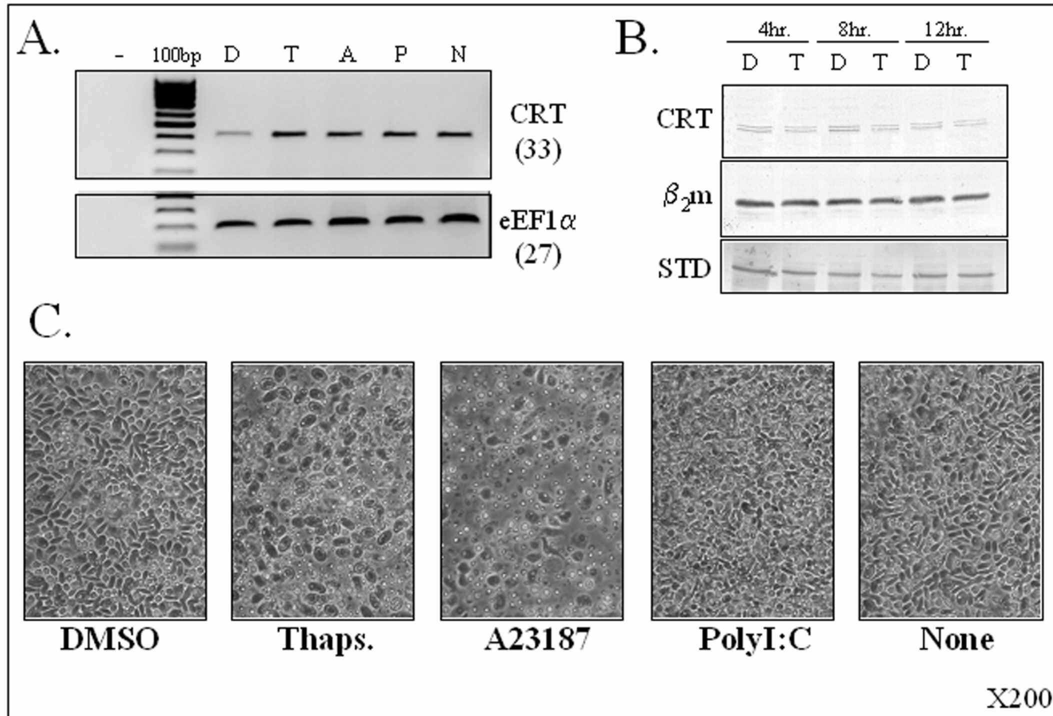

Figure 4-4. Effects of CHA on primary cultured trout PBL.

(**A**) RT PCR analysis of transcript steady-state levels for trout CRT and elongation factor alpha (eEF1α). PCR cycle numbers are indicated in brackets below (). Treatments are indicated above, as follows: DMSO (D), Thapsigargin (T), A23187 (A), PolyI:C (P) & No treatment (N). (**B**) Western blot analysis of protein steady-state levels during a 12hr. CHA treatment using Thapsigargin (T), while DMSO served as a vehicle control (D). Times are indicated above in hours for both control and treatment. Trout β_2m (~11kD) serves a reference protein, while Ponceau staining below (STD; ~98kD) serves as a measure of total protein banding between samples. (**C**) Phase contrast imaging shows cell populations at 12hr. following CHA treatment prior to analysis for gene expression above. Magnification is indicated below.

4.3.4. *In vitro* CHA challenge in a stable trout cell line

Due to the differences in PBL cell sub-population selection following CHA in a mixed primary culture of caudal blood, the more stable and homogenous rainbow trout cell line, RTS11 was employed to measure the effects of CHA upon endogenous CRT expression. As seen in trout PBL, CRT transcript appeared unchanged in RTS11 after 24 hours of CHA or polyI:C treatments (Fig. 4-5A), however transcript levels of trout COX-2 were up-regulated in cells treated with thapsigargin and calcium ionophore indicating that treatments were within physiologically responsive concentrations, as previously suggested for trout (Knight *et al.* 1993). Similarly, trout CRT protein steady-state levels in RTS11 remained unchanged following CHA treatment (Fig. 4-5B). Cell sub-population shifts and obvious cytological changes, observed in treated primary cultured PBL, were less apparent in RTS11 (Fig. 4-5).

4.3.5. CRT surface expression of cultured trout PBL

Affinity-purified trout CRT antisera demonstrated reactivity towards surface antigens of primary cultured trout PBLs (Fig. 4-6). Control treatments of the same cell preparations suggested that reactivity was specific to CRT as those treated with secondary antiserum alone showed no reactivity (Fig. 4-6). Excluding erythrocytes, which were negative for detectable CRT (Fig. 4-6), surface CRT expression appeared homogeneous throughout the mixed population of cells and not limited to a particular leukocyte type (Fig. 4-6). Qualitatively, primary cultured cells appeared to have enhanced surface expression following PHA treatment (Fig. 4-6), as previously shown in mammals (Arosa *et al.* 1999) however subsequent western blotting upon these same cell populations revealed that cellular protein steady-state levels appeared unchanged between the two treatment populations (Fig. 4-7).

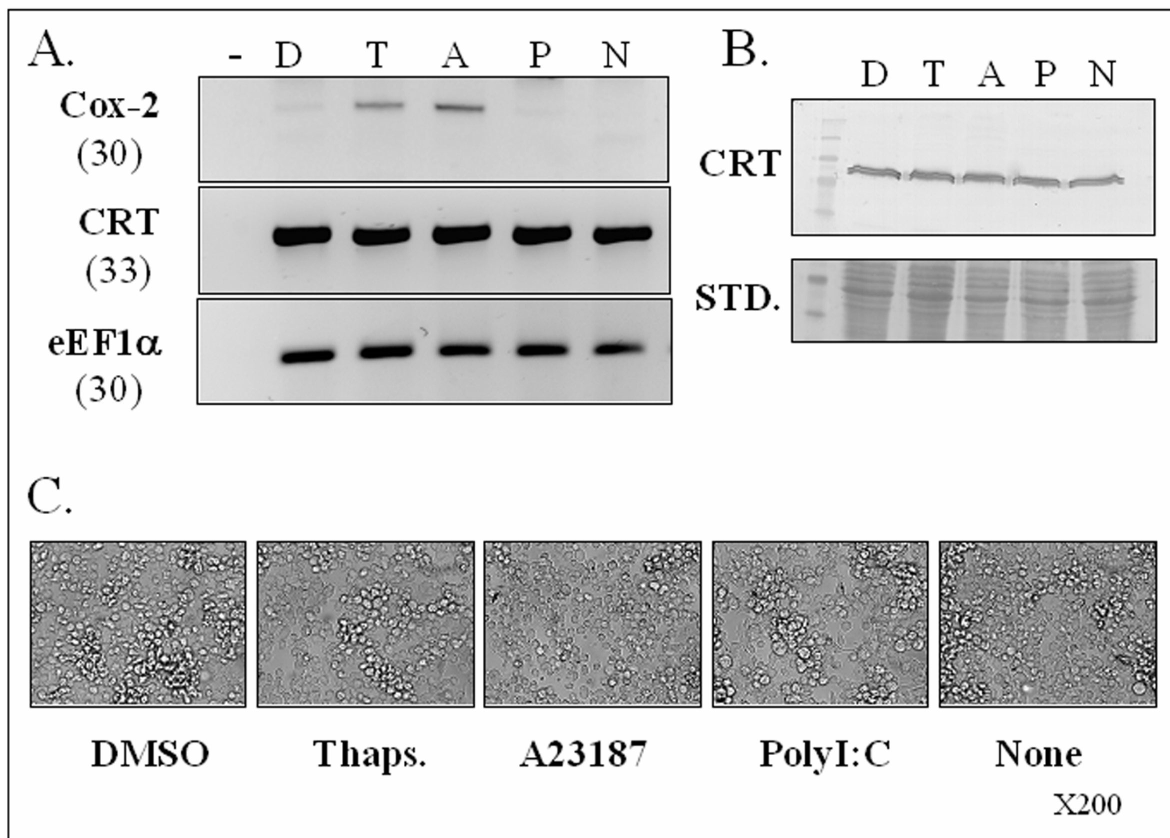

Figure 4-5. Effects of CHA on the rainbow trout macrophage cell line, RTS11.

(**A**) RT PCR analysis of transcript steady-state levels for trout COX-2, CRT and elongation factor alpha (eEF1α) following treatments as described for PBL exposures. PCR cycle numbers are indicated in brackets below (). (**B**) Western blot analysis of protein steady-state levels during a 12hr. CHA treatment using A23187 (A), while DMSO served as a vehicle control (D). Ponceau staining below (STD; ~98kD), prior to blocking, serves as a measure of total protein banding between samples. (**C**) Phase contrast imaging shows cell populations at 12hr. following CHA treatment prior to analysis for gene expression above. Magnification is indicated below.

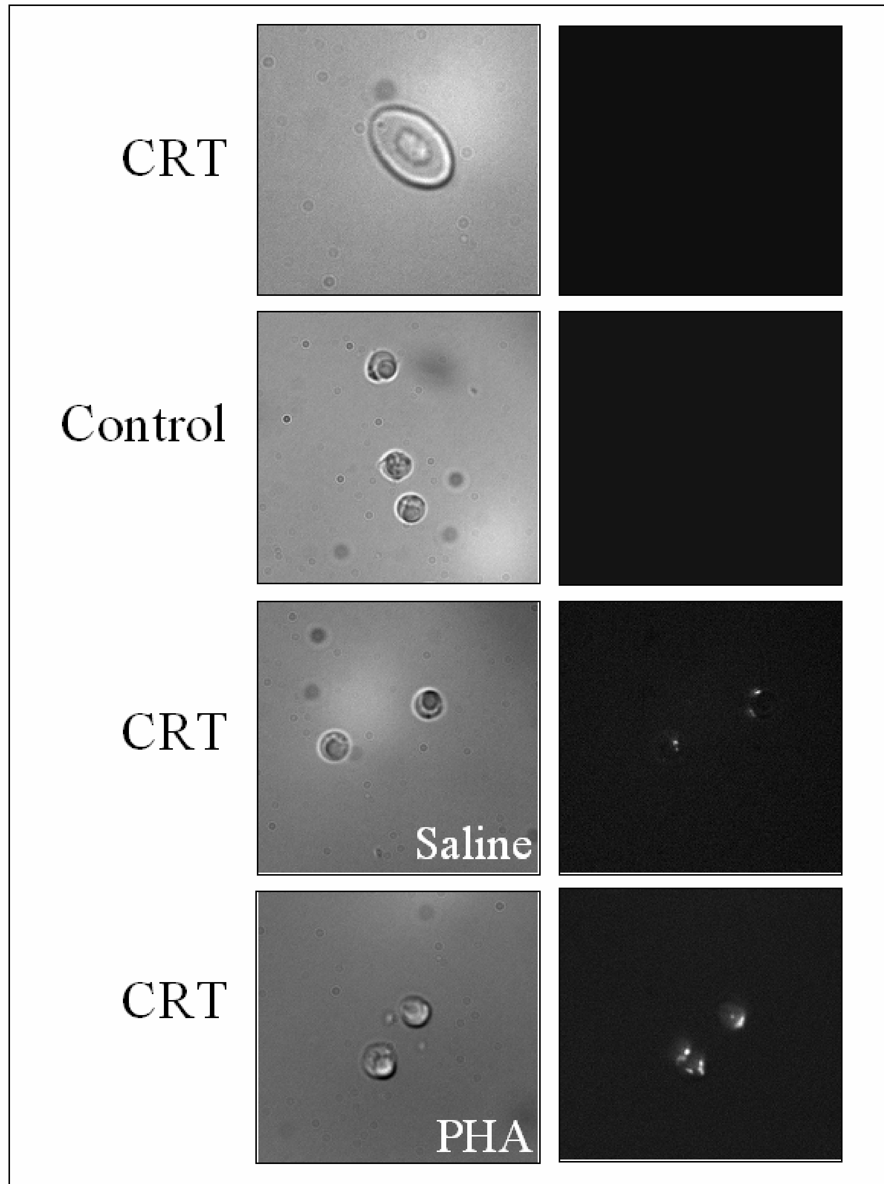

Figure 4-6. Surface calreticulin protein expression in trout PBL.

Epi-fluorescent detection of surface CRT in PBL preparations from trout caudal blood following 5 day *in vitro* treatment of PHA or saline control. Bright field images are shown on the left with corresponding fluorescence imaging on the right. Following collection, cells were probed using rabbit pre-immune serum (control) or trout CRT antiserum followed by secondary antibody coupled to FITC to assess surface antigen expression. Trout erythrocytes (top panel) showed no detectable CRT surface expression.

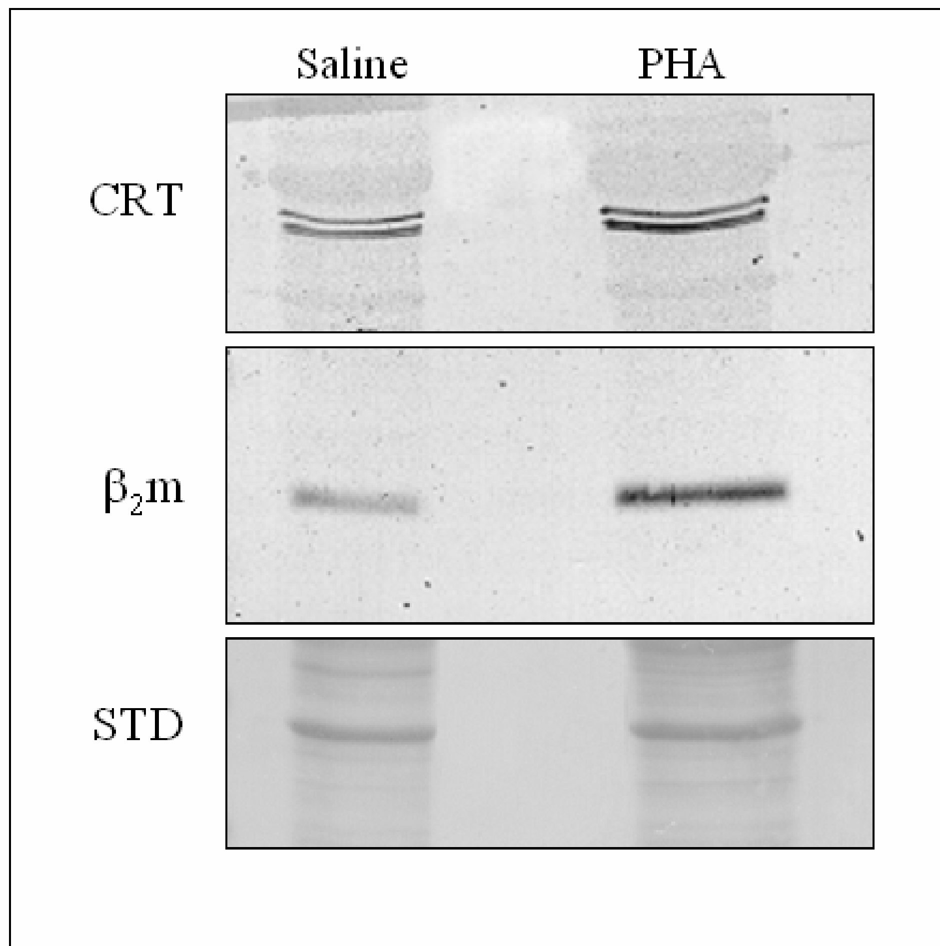

Figure 4-7. Cellular calreticulin protein levels in PHA-treated PBL.

Western blot analysis of total cell protein steady-state levels in PBL preparations following 5 day treatments with PHA and saline control. Blotted membrane was cut and separately probed for CRT and β_2m, which served as a reference protein, Ponceau staining below (STD), prior to blocking, serves as a measure of total protein banding between samples. (**A**) Trout red blood cells, treated as above failed to demonstrate detectable CRT surface expression. (**B**) Western blot analysis of total cell protein steady-state levels in PBL preparations following 4hr. treatments with PHA and saline control. The same blot was cut and probed with CRT or β_2m, which served as a reference protein.

4.4. DISCUSSION

Calreticulin is a highly conserved calcium binding protein found primarily in the endoplasmic reticulum of non-muscle cells. As the primary calcium modulator within the ER, CRT plays an essential role in embryonic development, calcium homeostasis and signaling. In mammals, CRT is strongly up-regulated in response to ER stress and CHA through its ERSE promoter elements (Yoshida *et al.* 2003). CRT's dual function as a key ER glycoprotein chaperone mediates its role in immune protein folding and function. Though considered an ER resident protein with its C-terminal ER retrieval sequence, CRT has been localized to sperm acrosomes (Nakamura *et al.* 1992) and the surface of activated T lymphocytes in mammals (Yoshida *et al.* 2003). Through the development of polyclonal antisera to recombinant rainbow trout CRT, we have found that the trout CRT protein is expressed primarily as a doublet with a relative mobility of ~60 kD in SDS-PAGE (Fig. 4-1), as similarly described in all other organisms to date (Michalak *et al.* 1999). Although trout CRT contains a potential N-glycosylation site, *in vitro* de-glycosylation revealed no shift in gel migration (Fig. 4-1) suggesting that this site is not utilized and that the doublet may represent an alternative post-translational modification or allelic differences in what is believed to be a single copy gene (Kales *et al.* 2004). Post-translational modification may provide a more likely explanation of protein doublet formation. In mammals, CRT has been shown to be a substrate of phosphorylation at serine and threonine residues. Whether the multiple reactive banding of trout CRT, observed in western blotting, is the product of differential phosphorylation remains unclear. Mammalian CRT, both native and recombinant, has the capacity for binding large amounts of calcium (Baksh *et al.* 1992). Differences in relative migration, resulting in doublet formation in polyacrylamide gels, have been previously demonstrated between calcium-free and calcium-bound CRT (Baksh *et al.* 1992; Balakier *et al.* 2002). The higher band observed in western blots of trout lysates may therefore represent reduced gel migration of a calcium-bound CRT due to increased positive charge. It is particularly interesting to note that western blotting reveals only the lower reactive band in sperm lysate, which is comparable to the lower relative band in all other tissues (Fig. 4-1B).

Based upon this observation, CRT may persist in the calcium-free state in trout sperm. Calreticulin has been identified in the sperm of several mammals, including rat (Nakamura *et al.* 1993), bull (Ho & Suarez, 2003), and human (Naaby-Hansen *et al.* 2001). In mammalian sperm, calreticulin together with calcium appears to be involved in regulating three important functions, capacitation, sperm motility and the acrosome reaction (Ho & Suarez, 2003; Naaby-Hansen *et al.* 2001). Rainbow trout sperm, like the sperm of most teleost fishes, do not undergo capacitation nor have acrosomes (Scott & Baynes, 1980) but their motility is regulated by calcium (Kho *et al.* 2004). Calcium-mediated sperm motility has implicated CRT in mammalian models and the role of calcium for sperm motility has been previously demonstrated in trout (Tanimoto *et al.* 1994). CRT may therefore serve a modulatory role in calcium-mediated sperm motility in trout. Studying calreticulin in teleost sperm could provide interesting comparative insights into calreticulin functions in reproductive biology.

Though first identified in rabbit skeletal muscle over 30 years ago, CRT expression in skeletal muscle has been shown to decrease through development (Koyabu *et al.* 1994) and plays only a minor role in calcium-binding, a role dominated by calsequestrin. Muscle used for this study represents dorsal skeletal muscle. Based upon western blotting, it appears that like mammals, trout CRT may play only a minor role in skeletal muscle, while conserving its role in calcium modulation for smooth & cardiac muscle such as the heart (Waisman *et al.* 1986).

As seen in mammals, the proximal promoter region of the trout CRT gene contains regulatory elements typical of genes transcribed by RNA polymerase II, including an Sp1 binding site at position +9. Though typically found in addition to upstream elements, functional Sp1 binding sites, downstream of a transcriptional initiation site, have been previously reported in humans (Pastorcic & Das, 1999) and may serve a similar function in trout CRT regulation. YY1, a zinc finger transcription factor has been shown to regulate numerous cellular and developmental genes (Shrivastava & Calame, 1995). YY1 sites have been identified as core elements of GRP78 genes in humans and found to be important for calcium-mediated UPR (Li *et al.* 1997), a role similarly described for ATF6 (Yoshida *et al.* 2003). The presence of a YY1 site in the trout CRT promoter suggests a similar role in fish.

As described in humans, the trout CRT promoter also contains two ERSE-like sequences (Fig. 4-3), which have been shown to be not only sufficient, but essential for CHA-mediated UPR exhibited in mammals (Yoshida *et al.* 2003). Interestingly, upon RT PCR and western blot analysis, rainbow trout CRT does not appear to be regulated in the same manner as mammals in response to *in vitro* CHA challenge. In mammals, CHA-mediated UPR is noted as early as 4 h and can reach a maximum by 16 h. In this report, trout cell challenges, using concentrations described in mammalian literature were extended to 24 h to account for the 19°C difference (37-18°C) in incubation temperature. Though direct comparisons to expression levels reported in the mammalian literature is complicated by these differences in incubation temperatures, the significant cytological changes observed suggest that the drug concentrations and incubation times employed in this study were sufficient for calcium homeostasis disruption. Furthermore, A23187 has been previously shown to induce production of prostaglandins (PGE_2) in trout macrophage (Knight *et al.* 1993) and cyclooxygenase 2 (COX-2; Zou *et al.* 1999). In this study, both A23187 and thapsigargin induced COX-2 expression by 12h, supporting effective dosage in these cells.

In mammals, viral infection can induce UPR (Isler *et al.* 2005) and has been shown to cause enhanced CRT expression (Zhu & Newkirk, 1994). In this report, the double-stranded RNA analogue, polyI:C, which triggers an antiviral response in fish cells (DeWitte-Orr *et al.* 2005), failed to elicit any significant change in CRT expression. Similar concentrations have been shown to elicit an antiviral state in RTS11 (DeWitte-Orr *et al.* 2005) which suggests that enhanced CRT expression, observed in mammals, may not be conserved or may be limited to live or intact virus. Considering the lack of change in CRT expression following the significant cytological changes observed in CHA-treated cells after 8 hours, and the induction of COX-2 transcript, we believe this lack of response in CRT expression levels suggests a different mode of regulation or function in fish.

Cellular localization may provide an alternative means of regulating CRT function. In mammals, CRT has been localized to the surface of activated T lymphocytes (Arosa *et al.* 1999). Similarly, CRT appears to be expressed on the surface of cultured trout PBL, the expression of which appears to be qualitatively enhanced following PHA treatment

(Fig. 4-6). PHA, a kidney bean lectin, is known to cause enhanced cell surface expression of CRT in association with free unfolded surface MHC in activated mammalian PBL (Mookerje *et al.* 1993). This enhanced surface detection is conserved in trout PBL, and may be due to protein recruitment rather than increased expression as overall protein steady-state levels in cell lysates appeared unchanged *via* western blotting (Fig. 4-7).

Based upon data presented here, trout calreticulin shares similar features to its mammalian homologue. Trout CRT is expressed in most tissues and cell types and demonstrates anomalous SDS-PAGE mobility primarily as a doublet. Despite the conservation of critical promoter elements, calcium homeostasis antagonists (CHA) do not appear to enhance the expression of trout CRT *in vitro* as demonstrated in mammals. Further investigations of the trout CRT promoter may elucidate the role of CRT in fish and facilitate a better understanding of the calcium-mediated ER stress response in mammals, while providing further insight into the regulation of this important protein. Current efforts include the use of reporter assays to further assess transcript steady-state levels upon various *in vitro* treatments along with co-immunoprecipitation.

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Chapter 5

Response of the rainbow trout monocyte/macrophage cell line, RTS11 to the water molds Achlya & Saprolegnia

ABSTRACT

The Saprolegniales are responsible for various fish mycoses worldwide and considered the most important fungi afflicting fresh water fish. Saprolegniosis leads to massive epidermal destruction and macrophage recruitment, yet little is known regarding the cytological response of their piscine hosts. The objective of this study was to explore the response of fish macrophage to members of the Saprolegniales using the rainbow trout monocyte/macrophage cell line, RTS11. After 48 hours in co-culture, RTS11 demonstrated chemotaxis, adherence and homotypic aggregation to both live and heat-killed fungal spores and mycelia. This aggregation was enhanced when using conditioned media from co-cultured RTS11 and *Achlya*, suggesting the presence of synergistic effectors of aggregation. Although fungal toxins were not evident, as cells remained viable throughout fungal overgrowth, phagocytosis was inhibited due to large fungal spore size, allowing these molds to evade macrophage defenses. Although class I MH and other viral response genes showed no significant change in expression, calreticulin and interleukin-8 were moderately up-regulated implicating calcium modulation and chemotactic response, respectively. Cyclooxygenase (COX-2) and the cytokines IL-1β and TNFα were strongly up-regulated in the presence of *Achlya,* while components of class II Major Histocompatibility (MH) appeared down-regulated, suggesting fungal interference of immune function. Previous studies have shown an increased dependence of macrophage in immune function at low temperatures; based upon data presented here, this reduction of macrophage class II MH receptor expression and inability to phagocytose spores may limit host response thereby providing increased susceptibility to these opportunistic pathogens.

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5.1. INTRODUCTION

The Saprolegniales are an aquatic order of the Oomycota, (*Egg-fungi*) which includes notorious terrestrial pathogens such as the Irish potato blight fungus, *Phytophthora infestans*, the grapevine downy mildew, *Plasmopara viticola* and mammalian "swamp cancer", *Pythium insidiosum*. As their name implies, the Saprolegniales are considered mainly saprophytic, serving simply as organic decomposers of aquatic ecosystems, however like their terrestrial counterparts several are considered highly opportunistic pathogens. Although their membership in the Kingdom Fungi is still of much debate, this group is considered to be the most important fungal group affecting both wild and farmed fishes (Bruno & Wood, 1999). Species of *Aphanomyces*, *Saprolegnia* and *Achlya*, all members of the Saprolegniales, are major pathogens of many fish species (Jeney & Jeney, 1995). *Achlya debaryana* was demonstrated as the causative agent of epizootic mycoses of channel catfish in India (Khulbe *et al.* 1994). In that same year, *Saprolegnia* was isolated as the cause of "winter kill" in Mississippi catfish farms (Bly *et al.* 1992) and more recently, caused an epizootic mycosis in Japanese salmon farms, further demonstrating this organism's ubiquity (Hatai & Hoshiai, 2001). Fish serve as an excellent source of protein worldwide. To satisfy global demand, a seven-fold increase in fish production is anticipated within the next fifteen years, yet because natural sources are being quickly exploited, aquaculture is becoming the chief source of this valuable resource (Fletcher *et al.* 1999). Unfortunately, current farming conditions often lead to increased frequency of disease. Saprolegniosis outbreaks have led to considerable economic damage and many attempts have been made to deal with this pathogen.

These pathogens can be found in almost any body of fresh water, making the potential for disease difficult to avoid and further highlighting the importance in understanding their biology. They are also, however, one of the easiest fungi to isolate and cultivate, allowing them to be readily studied under laboratory conditions. Previous histopathological studies of saprolegniosis outbreaks have reported extensive tissue destruction and hyphal invasion into underlying tissues, musculature and organs (Neish, 1977).

Though still considered one of the most important fungal pathogens of fish (Torto-Alalibo *et al.* 2005), little is known of the cytological and immunological response of their piscine hosts. Previous studies have shown that *in vivo* challenges using a fungal inoculum following a variety of stressors leads to significant epidermal destruction and macrophage recruitment. In response to *Aphanomyces invadans*, the causative agent of low-temperature-related epizootic ulcerative syndrome (EUS), primary cultured rainbow trout head kidney macrophages formed multinucleated giant cells and aggregates surrounding the hyphal tissue and phagocytosed fungal spores (Miles *et al*. 2001). At low spore concentrations ($<10^3$ spores/mL), macrophage phagocytosis was able to inhibit spore germination, however higher spore concentrations rapidly led to fungal overgrowth and spore germination was noted within macrophage phagosomes (Thompson *et al*. 1999). Studies have revealed an increase in macrophage activity at lower temperatures, as previously described by Chinabut *et al.* 1995. Increased respiratory burst was also demonstrated at low temperatures; however, this was prevented in the presence of the mold (Miles *et al*. 2001) leading the authors to suggest the possibility of fungal interference with superoxide production, as previously described for the fish pathogen *Yersinia* (Stave *et al.* 1997) and the potato blight fungus, *P. infestans* (Doke, 1983). In mammals, both macrophage and neutrophils serve as the major lines of cellular defense to systemic and opportunistic fungal infections through attachment and phagocytosis of the invader, and the stimulation of other immune cells, including T & B lymphocytes (Fromtling & Shodomy, 1986). Unlike mammals, fish neutrophils account for only a small portion of total leukocyte number (Afonsa *et al*. 1997) placing a significant burden of initial immune responses on piscine macrophage and further highlighting their importance in responding to opportunistic invaders. To further understand the physical and molecular response of fish macrophage to fungal pathogens, this study examined the response of the rainbow trout monocyte/macrophage cell line, RTS11 (Ganassin & Bols, 1998) to the presence of Saprolegniales pathogens.

5.2. MATERIALS AND METHODS

5.2.1. Fungal cultures

Achlya bisexualis and *Saprolegnia parasitica* were kindly provided by Dr. N. Money, Miami University and Dr. D. Patchett, North Carolina University at Wilmington and maintained on corn meal agar (Sigma, St. Louis, MS) stored at 4^oC. For spore inoculum preparations, agar plugs were excised at the periphery of actively growing mycelia and transferred to petri plates containing sterile water supplemented with 25mM $CaCl_2$/KCl (Griffin, 1978). After one week at ambient temperature (22^oC), cultures were lightly vortexed and the liquid medium, containing encysted spores, removed and stored at 4^oC prior to use. For heat-killed inocula, spore preparations and/or fresh mycelia from liquid culture were boiled for 10 minutes and cooled prior to use.

5.2.2. Calcofluor staining of fungal inocula

To facilitate fungal detection in co-culture conditions, viable and heat-killed inocula were treated for 10 minutes with 0.1% Calcofluor white M2R in 2M sodium phosphate buffer; pH 8.0 (Fischer *et al*. 1985) then washed with distilled water and resuspended in $CaCl_2$/KCl solution prior to use. Staining was employed either prior to inoculation or after fungal-macrophage co-culture.

5.2.3. Cell culture

RTS11, a continuous rainbow trout macrophage-like cell line, originally isolated from a long-term spleen haemopoietic culture (Ganassin & Bols, 1998) was maintained at 18^oC in 25cm^2 tissue-culture-treated flasks (Nunc), in Leibovitz's L-15 media (Gibco) supplemented with 1% penicillin/streptomycin and 15% fetal bovine serum (FBS). Confluent flasks were passaged monthly by splitting cells and conditioned media with equal volume of fresh media into two separate flasks. RTG-2, a rainbow trout gonadal fibroblast cell line was obtained from the American Type Culture Collection (ATCC) and cultured as previously described (Wolf & Quimby, 1962) in Leibovitz's L-15 media (Gibco) supplemented with 1% penicillin/streptomycin and 10% FBS.

5.2.4. *In vitro* challenge

For *in vitro* co-culture challenges, RTS11 cells were plated at concentrations of 1.0 & 2.0 x10^7 cells/mL in 60mm treated cell culture dishes (Corning) with L-15 media supplemented with 1% penicillin/streptomycin and 15% fetal bovine serum (FBS) and allowed to attach overnight at ambient temperature (22°C) prior to the addition of spore inocula. RTG-2 cells were plated at 2.0 x10^6 cells/plate in L-15 supplemented with 1% penicillin/streptomycin and 10% FBS and kept overnight at ambient temperature prior to fungal inoculation. In all cases, duplicate plates were treated with $CaCl_2$/KCl media lacking spores as a vehicle control, while plates containing media, but lacking rainbow trout cells, were inoculated as fungal controls. For conditioned media treatments, media from 48 hour cell/fungal co-cultures and controls, as described, were filtered through 0.2µm syringe filters and mixed with equal volume of fresh media on separate 24 hr. RTS11 cultures.

5.2.5. Microscopic examination and recording

Cultures were regularly examined using a light microscope throughout challenges to assess cellular response and fungal growth. Plates were visualized using a Zeiss Axioskop 2 Plus, equipped with an ultraviolet (u.v.) filter and a Zeiss Axiovert 35, for higher magnification bright-field viewing. Images were digitally recorded using Northern Eclipse imaging software (Empix Imaging).

5.2.6. Reverse transcriptase (RT) PCR analysis of gene transcripts

Adherent cells were scraped and collected with media along with non-adherent cells and fungal co-culture, where applicable, washed in 1X PBS and stored at –80oC prior to cDNA synthesis. Total RNA was isolated from frozen cell pellets using Trizol (Invitrogen) according to manufacturer's instructions. RNA yield was determined by absorbance at 260nm and 5µg of total RNA was diluted in diethylpyrocarbonate (DEPC)-treated water for cDNA synthesis using oligo dT and First strand cDNA Synthesis Kit (Fermentas) according to manufacturer's instructions. Subsequent PCR was performed for 30 cycles using primers designed against published sequence data at annealing temperatures indicated in Table 1 to determine relative transcript steady-state levels.

Table 5-1. Oligonucleotide sequences employed for RT PCR analysis.

Primers were designed against published sequence data acquired from GenBank. Target cDNAs are shown on the left and primer sets (sense and antisense) are shown in a 5'-3' orientation. PCR annealing temperatures are indicated on the right. All reactions were performed for 30 cycles.

Table 1: Oligonucleotide sequences used for RT-PCR

target	primer	oligonucleotide sequence	annealing temp.
MHC I	RTCIA3F1	5'-CTACGGCAAAGACACTTTGG-3'	58°C
	RTCIABR1	5'-GTGGGAGCTTTTTTGGAAGG-3'	
β2m	Onmy B2MF1	5'-TGTCAATCGTTGTACTTGGG-3'	58°C
	OnmyB2MR1	5'-CTTCAGGTGGCGGACTCTGC-3'	
CRT	RTCRTF1	5'-AGACATCTGTGGCTACAGC-3'	54°C
	RTCRTrev1	5'-ACTCTCCCTGATACTCCGG-3'	
STAT-1	RTSTAT1for	5'-CAGGTCGACCAGCTCTACG-3'	58°C
	RTSTAT1rev	5'-TCTTCTGCTCCTCCTTCAGG-3'	
IFN	RTIFNF1	5'-TGCCCCAGTCCTTTTCC-3'	58°C
	RTIFNR1	5'-TACATCTGTGCCGCAAGG-3'	
Mx2	Mx2 forward	5'-CTTGGTAGACAAAGGCACAGAGGA-3'	65°C
	Mx2 reverse	5'-AAGTTCTTTCCAGAGCGATCCA-3'	
Mx3	Mx3 forward	5'-ATGCCACCCTACAGGAGATGAT-3'	53°C
	Ms3 reverse	5'-CCACAGTGTACATTTAGTTG-3'	
CIIα	OnmyDAAF3	5'-TAGGTAATCTGGGGATATGC-3'	52°C
	OnmyDAAR	5'-GCTCAGTAAGGGCCTTGTGC-3'	
CIIβ	OnmyCIIBmat645-626	5'-CATTAGCAGGACTGATCTAC-3'	52°C
	mykissCIIBantisense	5'-GGGTGTGTACTCCAGGTGGG-3'	
S25-7	INVS257 specific sense	5'-GGAGAAGCCCCCTGCACCCA-3'	52°C
	INVS257 specific antisense	5'-ATCATCCTGGGGAAAGCTGC-3'	
INVX	INVX sense	5'-ATCAGAGGAGGCCATCTTAC-3'	54°C
	INVX RTPCR antisense	5'-GTCTGGTTCACATCTCTTGG-3'	
14-1	INUL14-1 sense	5'-AGCTGCATGTGCCCATGAAC-3'	54°C
	INVL14-1antisense	5'-CTTGGTTGGCCTAATCTCAG-3'	
COX-2	F6	5'-ATCCTTACTCACTACAAAGG-3'	53°C
	R3	5'-GCTGGTCCTTTCATGAAGTCTG-3'	
IL-1β	OnmyIL1β sense	5'-CCTGATGAATGAGGCTATGG-3'	53°C
	OnmyIL1β antisense	5'-TTCCTGAAACTGGCAGACTC-3'	
TNF α	OnmyTNFalpha F1	5'-TGGCTATGGAGGCTGTGTGGGGTC-3'	53°C
	OnmyTNFalpha R1	5'-GCCTTCGCCAATTTCGGACTCAGC-3'	
IL-8	OnmyIL8 CDS sense	5'-CATCAGAATGTCAGCCAG-3'	52°C
	OnmyIL8 CDS antisense	5'-CCCTCTTCATTTGTTGTTGG-3'	
CD9	RtCD9for	5'-AGCTGTGCAAGTGTTTCCTC-3'	53°C
	RtCD9rev	5'-CAAGGCACCAATGAGTCCAC-3'	
iNOS	iNOS forward 1	5'-TCCCAGCATGCCCTTTGTCTC-3'	57°C
	iNOS reverse 1	5'-ACTCCCTGGGCCATGTTCACA-3'	
CK1	CK1-AS2	5'-TGGAAGATGACAGCGTTGAGG-3'	57°C
	CK1-S3	5'-CTGGCTGCTCTGTTCTCTCT-3'	
CK2	CK-2 gDIG sense	5'GCAGAAAAGCTGGTGTCGTG-3'	57°C
	CK-2 antisense	5'GGAAGGTACGGATGGAGAAG-3'	
C5aR	ND29 SP1	5'-CCTGGACCGCTTTCTGCTGG-3'	57°C
	ND29 SP2	5'-CATAGAGCAGAGGGTTGAGG-3'	
eEF1α	R TROUT EFTU-1 SENSE	5'-GAGTGAGCGCACAGTAACAC-3'	58°C
	R TROUT EFTU-1 ANTISENSE	5'-AAAGAGCCCTTGCCCATCTC-3'	

Thermal cycling parameters, for all reactions, were as follows: 95°C for 5min., (95°C for 45s., X° annealing temperature for 45 s. (see Table 1), 72°C for 45 s.) at 30 cycles, 72° for 10 min. Elongation factor alpha (eEF1α) served as an internal standard for equal cDNA template loading (See Eftu-1 in Hansen & Strassburger, 2000; Thompson, 1988).

5.2.7. Western blot analysis of cellular protein expression

Cell pellets were collected as above, lightly vortexed and left on ice for 30 minutes in 500 µL of protein lysis buffer (1% NP-40, 150mM NaCl, 10mM Tris; pH 7.4) supplemented with 2mM PMSF and protease inhibitor cocktail (Sigma). The crude lysates were cleared by centrifugation at 4°C for 10 minutes at 15 000 rpm and the supernatant analyzed by Bradford method to determine total protein concentration. Approximately 100 µg of total protein was separated by 15% acrylamide SDS-PAGE and electroblotted to nitrocellulose membrane. Total protein transfer was assessed using 0.2% Ponceau S (Fisher) in 5% acetic acid and recorded using a flatbed scanner then washed and blocked for 1 hour in TBS-tween containing 5% skim milk powder. Blots were then probed for 1 hour at room temperature in blocking buffer containing affinity-purified rabbit antisera previously developed in our laboratory against recombinant forms of rainbow trout Major Histocompatibility (MH) sub-units (Kales *et al*. 2006; Nath *et al*. 2006). For β_2m expression, blots were washed and probed using goat anti-rabbit alkaline phosphatase (Sigma) at 1: 30 000 for 45 minutes prior to detection using NBT/BCIP and digitally recorded on a flatbed scanner. For Class II MH expression, replicate blots were alternatively probed using goat anti-rabbit horseradish peroxidase at 1:2500 and detected using ECL Plus Western blot detection system (Amersham) according to manufacturer's instructions and digitally recorded using a Fluorchem 8000 (Alpha Innotech).

5.3. RESULTS

5.3.1. Cellular effects of fungal-macrophage co-culture

Within 48 hours of co-culture, RTS11 formed adherent aggregates along the growing mycelia of *Achlya* (Fig. 5-1). Macrophage could be seen aggregating around the heavily melanized chlamydospores (Fig. 5-1A) and along hyphal filaments (B). Aggregation along *Achlya* hyphae appeared to non-uniform, as some regions appeared devoid of macrophage clustering (Fig. 5-1 C&D). Clustering occurred above the plane of original adherent cells (Fig. 5-1 C) suggesting chemotactic motility towards the hyphal filaments. *Saprolegnia* growth appeared more prolific and attracted macrophage clustering along most of the hyphal surface within 48 hours (Fig. 5-1 E&F). Although it was clearly evident in the absence of FBS, macrophage adherence to the fungus was enhanced in the presence of FBS (data not shown). Due to the ease of spore production, *Achlya* cultures were employed for further study in the presence of FBS. Calcofluor staining facilitated hyphal filament and spore identification in co-culture (Fig. 5-2A&B) and appeared to have no inhibitory effects upon spore germination or macrophage adherence (Fig. 5-2C&D). Fluorescently labeled cellulose demonstrated a gradient distribution along the growing hyphal filament following germination suggesting that cellulose, which originally formed the fungal spore wall was reutilized along the growing germ tube and extending filament (Fig. 5-2D). The trout macrophage cell line also demonstrated adherence to heat-killed mycelia (Fig. 5-3A&B) and heat-killed spores (Fig. 5-3C) suggesting that the pathogen released a heat tolerant chemotropic factor. In the presence of the mold, cells adopted a flattened, spreading morphology suggesting a shift to a mature macrophage population (Fig. 5-3C&D; Ganassin & Bols, 1998).

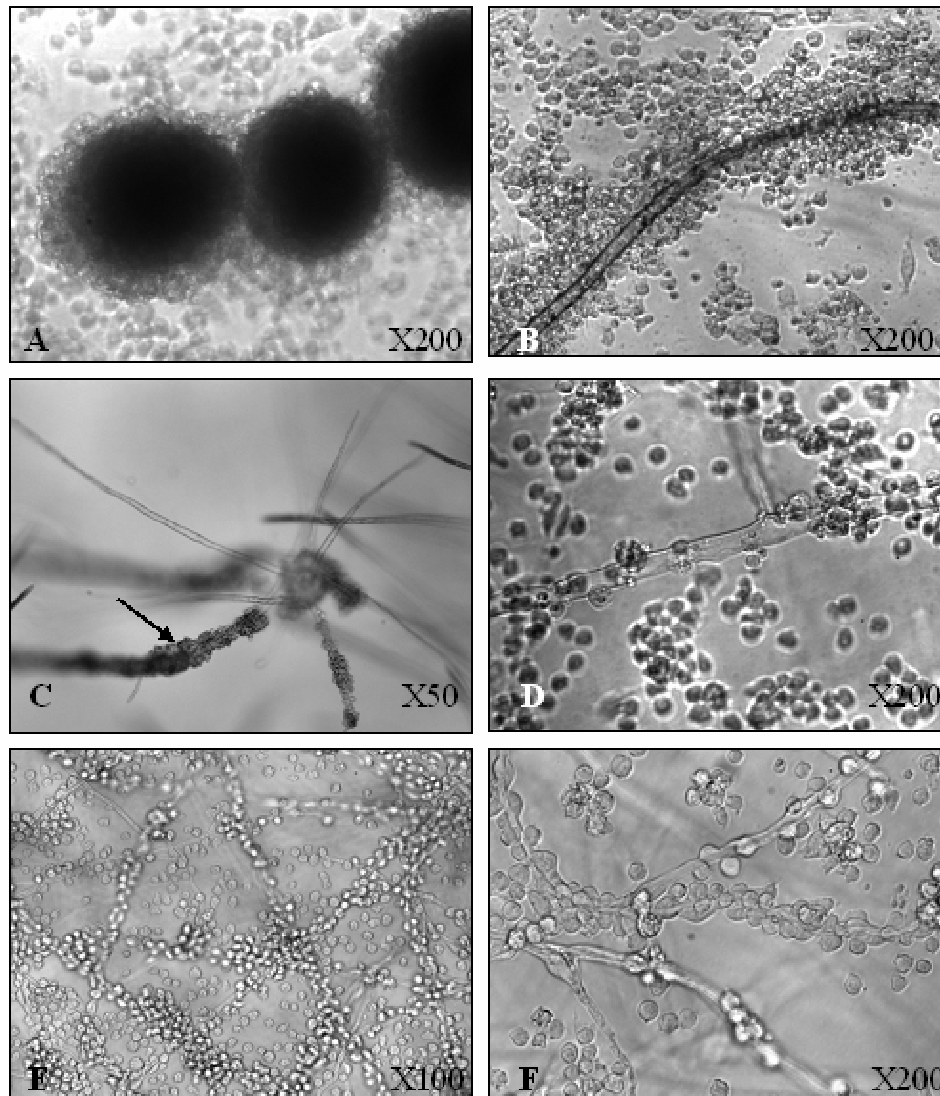

Figure 5-1. RTS11 adherence to cultures of *Achlya bisexualis* and *Saprolegnia parasitica*.

RTS11 cells were incubated with appropriate spore inoculum at ambient temperature for 48 hours. In panel **A**, macrophage adhere to *Achlya* chlamydospores. **B**. Macrophage adherence to *Achlya* hypha. **C**. Macrophage attachment above the plane of original adherent cells suggesting chemotropism and/or attachment by cells originally in suspension. **D**. Non-uniform adherence of macrophage to *Achlya* hypha. In panels **E&F**, macrophage adhere to hyphae of *Saprolegnia parasitica*. Magnification is indicated at the bottom right in each panel.

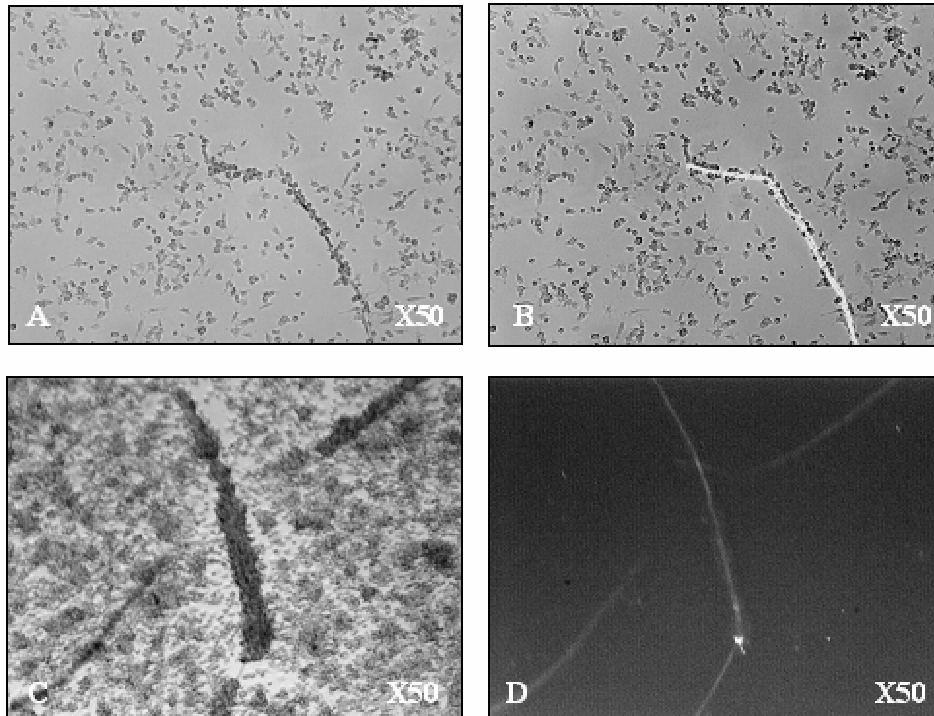

Figure 5-2. Calcofluor staining of fungal hyphae in trout macrophage co-culture.

In panel **A**, bright-field microscopy illustrates macrophage adherence to *Achlya* hypha. **B**. Merged bright-field and fluorescence imaging of hyphal filament at the site of macrophage aggregation, where staining was performed following 48 hr. co-culture. Bright-field **(C)** and corresponding fluorescence imaging **(D)** of macrophage aggregation 48 hr. after inoculation using pre-stained spores. Fungal spore germination was not inhibited by calcofluor pre-treatment. Fluorescence along growing hyphae indicated reutilization of spore coat cellulose. All images shown at 50X magnification.

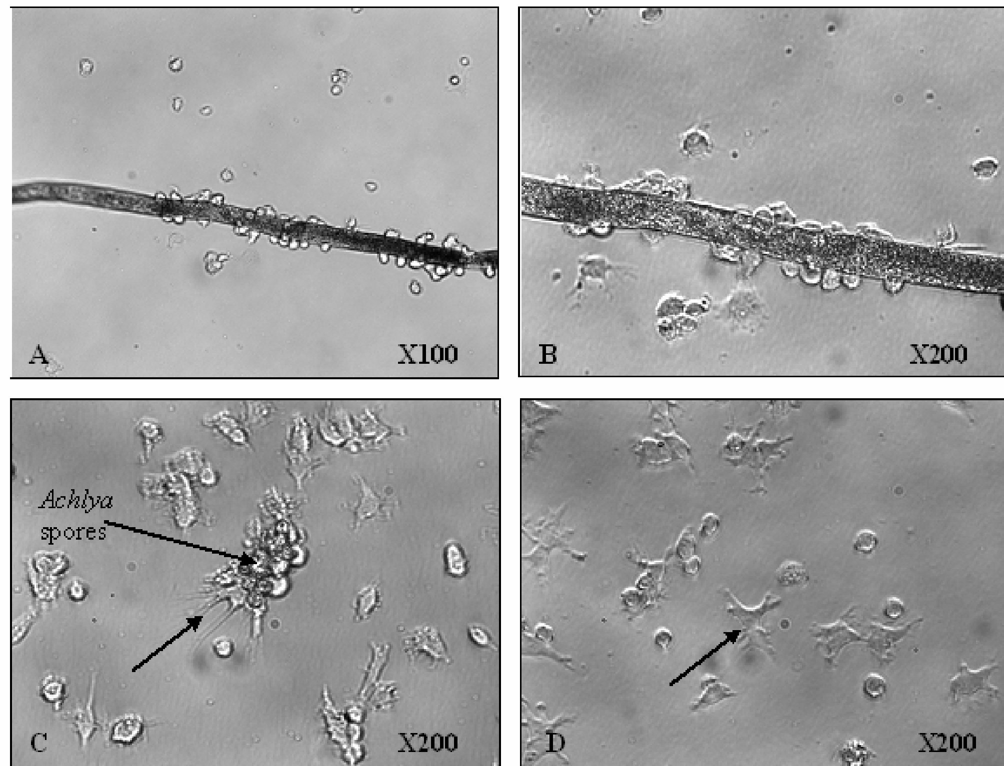

Figure 5-3. RTS11 response to heat-killed *Achlya* mycelium.

Panels **A&B** illustrate magnified view of macrophage adherence to heat-killed *Achlya* hypha. **C&D**. Macrophage demonstrate spreading morphology in the presence of heat-killed *Achlya* spores. Magnification indicated at the bottom right.

To address whether or not cell adherence to the mold was simply due to the availability of a solid surface or to cellulose, a main component of Oomycete cell walls, adherent macrophage cultures were exposed to sterile cotton fibres and uv-sterilized monofilament nylon fishing line for 48 hr. Macrophage failed to demonstrate any detectable adherence or aggregation to either the cellulose fibres (Fig. 5-4A) or the solid nylon substrate (data not shown). To assess if cell adherence to fungal hyphae was macrophage specific, the rainbow trout gonadal fibroblast cell line, RTG-2 was grown in co-culture, but did not demonstrate aggregation or adherence towards the growing fungus, which appeared to readily penetrate the confluent layer of these fibroblasts within the 48 hr. challenge (Fig. 5-4B). Unlike a previous study using *Aphanomyces* (Miles *et al*, 2001), evidence of macrophage phagocytosis of fungal spores was not detected. Cytotoxicity due to the presence of potential fungal toxins was also not evident as macrophage viability was not reduced during fungal overgrowth (data not shown). In cultures using higher RTS11 concentrations, (2.0 $x10^7$ cells/mL), macrophage demonstrated homotypic aggregation in the presence of the fungus or its conditioned media (Fig. 5-5). Homotypic aggregation (HA) appeared greatest in conditioned media obtained from co-culture rather than from RTS11 or *Achlya* grown in media separately, suggesting a synergistic effect between factors produced by both the fungus and the macrophage (Fig. 5-5B). RTS11 treated with conditioned media from RTS11 grown alone that therefore did not contain any fungal components showed little to no HA (Fig. 5-5; left panels). Treatment of RTS11 with live *Achlya* resulted in rapid overgrowth of cultures and therefore required heat-killed *Achlya* for longer exposures. Macrophage incubated with heat-killed *Achlya* for 10 days also demonstrated HA, further supporting the theory that the fungus produces a heat tolerant aggregation factor (Fig. 5-5C).

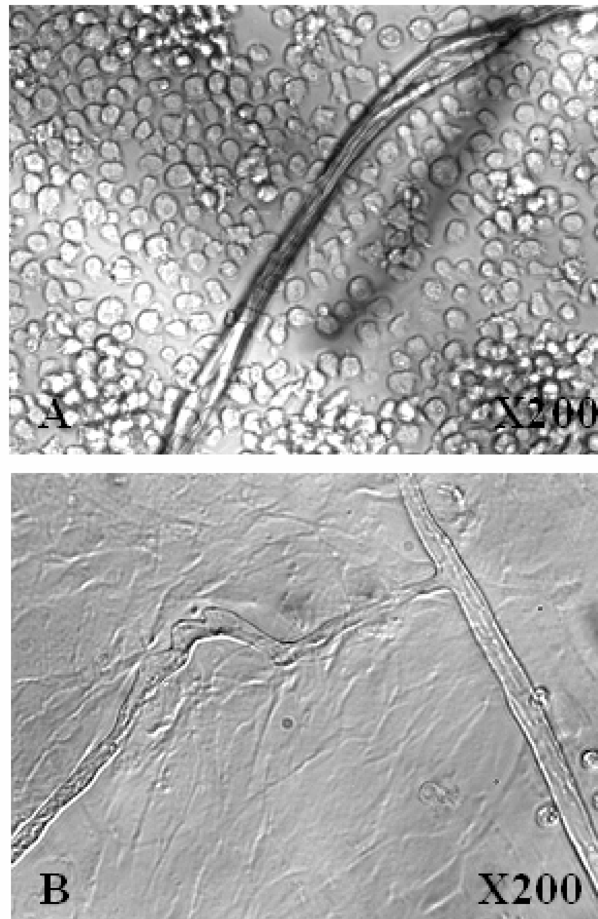

Figure 5-4. Testing of adherence and aggregation by cell-type and substrate.

RTS11 demonstrates no evidence of chemotropism or adherence, after 48 hr., to sterile cotton (cellulose) fibres. **(B)** RTG-2, a rainbow trout gonadal fibroblast cell line after 48 hr. in the presence of live *Achlya* culture. *Achlya* readily penetrate the confluent layer of fibroblasts with no evidence of cell chemotropism or adherence.

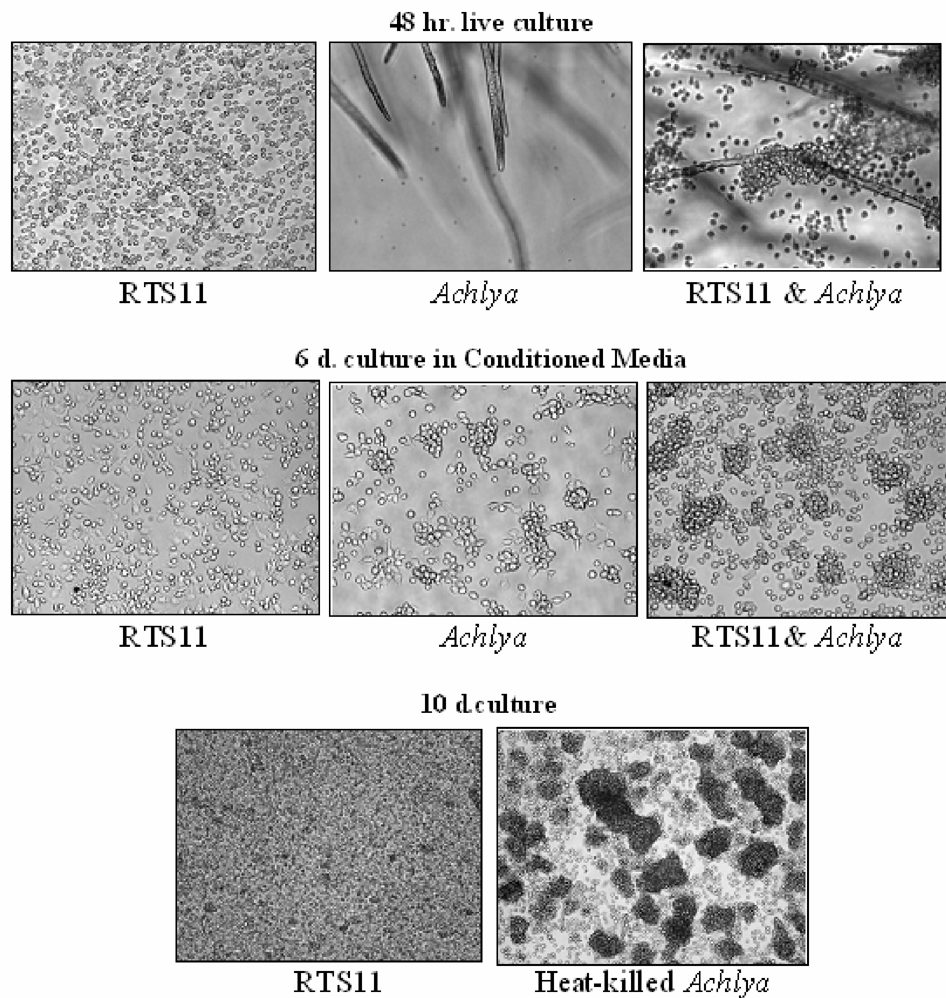

Figure 5-5. HA in RTS11 by *Achlya* cultures and their filtrate.

Top panels illustrate 48 hr. cultures of RTS11 alone, *Achlya* alone and RTS11 wth live *Achlya*. Media from each of the above cultures was filtered and diluted 1:1 with fresh media to serve as conditioned media for freshly plated cells. **Middle panels** illustrate macrophage following 6 d. in conditioned media collected from top panel cultures. Homotypic aggregation was greatest in cultures grown in conditioned media from co-culture (**middle right panel**) suggesting synergistic effectors. **Bottom panels** illustrate RTS11 after 10 days alone (**bottom left panel**) or in the presence of heat-killed spore inoculum (**bottom right panel**). Cells in lower panels were plated at highest density to enhance the density-dependent homotypic aggregation. Magnification X100.

5.3.2. Effects of fungal co-culture on macrophage gene expression

In order to assess the molecular response of the macrophage cell line after 48 hours in co-culture with *Achlya*, gene transcript levels were examined by reverse transcriptase (RT) PCR. Basal transcript levels were determined using templates from RTS11 cultured alone, while templates from *Achlya* cultures alone served as a negative control (Fig. 5-6R; A, respectively). For comparison between treatments, and to ensure equal loading of templates, elongation factor alpha (eEF1α) transcript levels were used as an internal standard as previously described (See Eftu-1 in Hansen & Strassburger, 2000; Thompson, 1988). The expression of genes encoding the class I Major Histocompatibility (MH) receptor sub-units (β_{-2}-microglobulin & MH I) appeared unchanged, while expression of the gene encoding their chaperone, calreticulin, appeared moderately up-regulated (Fig. 5-6; R/A). STAT-1, a signaling pathway component of various extracellular ligands appeared unchanged as did that of type 1 interferon (IFNα) while genes encoding the antiviral proteins, Mx2 &3 demonstrated little to no detectable induction in the presence of the fungus. The class II MH receptor typically involved in immune responses to exogenous antigens including bacteria and fungi, appeared to be down-regulated in the presence of the fungus. The genes encoding the two sub-units of the class II MH receptor, class II α and class IIβ, as well as those encoding their invariant chain chaperones (S25-7 & INVX) demonstrated decreased transcript steady-state levels in the presence of the fungus. Transcript levels of the invariant chain-like peptide, 14-1, yet to be fully characterized, appeared unchanged, further adding to the mystery of its function while the gene encoding inducible cycoloxygenase (COX-2), an enzyme involved in prostaglandin synthesis, was strongly up-regulated in the presence of the mold indicating a pro-inflammatory response. In addition, the pro-inflammatory cytokine genes, interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNFα) were also strongly induced in the presence of the fungus. In contrast to this, interleukin-8 (IL-8) showed only moderate up-regulation, while those encoding CD9, a receptor involved in cell-to-cell adhesion, and the inducible nitric oxide synthase (iNOS) appeared slightly down-regulated in the presence of the fungus. The chemokine gene, CK1 produced no detectable levels of transcript with or without the presence of the mold, while another trout chemokine gene, CK2 and and that encoding the complement receptor, C5aR were expressed but showed little to no change in gene expression in the presence of the mold.

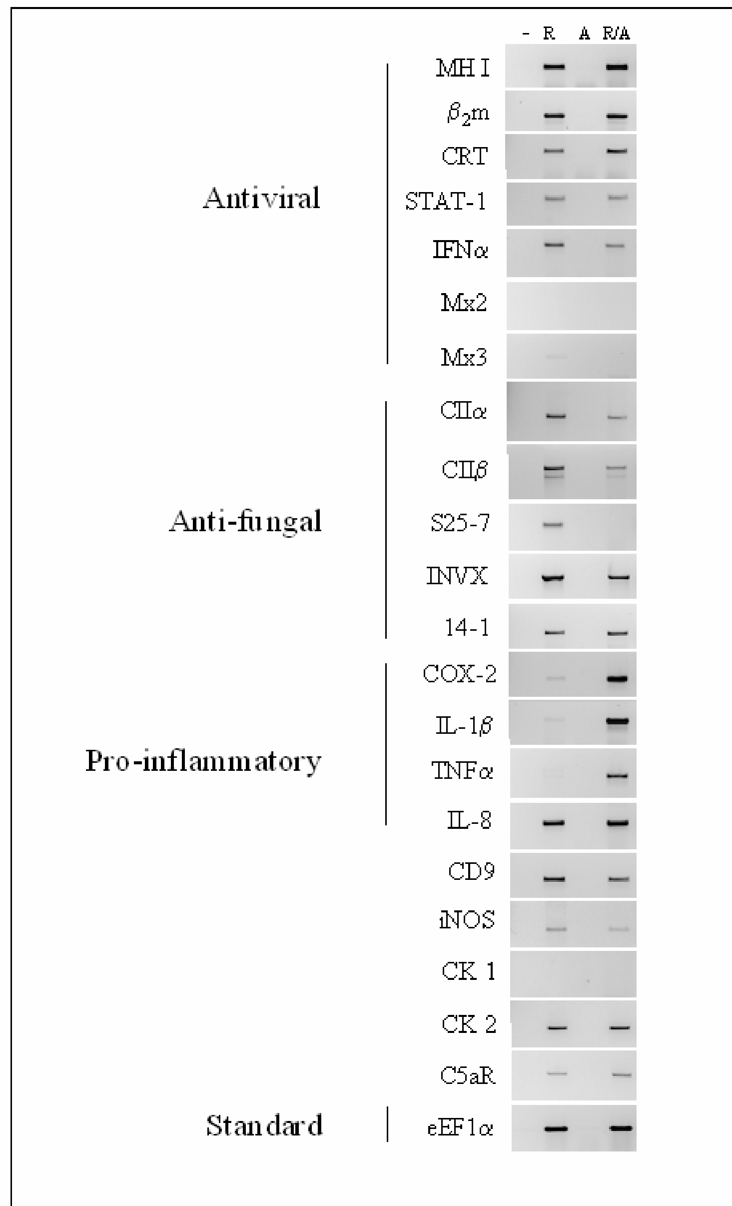

Figure 5-6. RTS11 gene expression in the presence of *Achlya.*

Target cDNAs are indicated on the left from RTS11 grown alone for 48 hr. **(lane R).** *Achlya* grown alone shows no amplification of target trout cDNAs **(A).** In **lane R/A**, cDNA template was derived from RTS11 grown for equal time in the presence of live *Achlya* culture. Genes are grouped by function as indicated on the left. PCR reactions included water in place of cDNA template to serve as negative controls, indicated with a *minus sign* **(-)**. Equal amounts of total RNA, determined by absorbance at 260nm, were used in cDNA synthesis reactions. Band intensity serves as a measure of original transcript abundance relative to the internal standard, eEF1α as previously described by Hansen & Strassburger, 2000.

The transcript levels of elongation factor alpha (eEF1α), which was used as an internal standard of RNA integrity and cDNA template loading (Fig. 5-6; Eftu-1 in Hansen & Strassburger, 2000), suggested equal loading due to similar band intensities between lanes.

5.3.3. Effects of fungal co-culture on macrophage protein expression

To determine if these changes in gene expression affected protein levels, cellular protein steady-state levels were assessed by western blotting using affinity-purified anti-trout β_2m and anti-class II MH alpha polyclonal antibodies previously developed in our laboratory. Total protein banding, assessed by Ponceau S staining, served as a standard for equal protein loading between samples (Fig. 5-7 STD). As seen in the transcript steady state level study described above, the class I MH receptor sub-unit, beta-$_2$-microglobulin, showed no detectable change in the presence of the fungus (Fig. 5-7). Although moderately down-regulated at the transcript level, the class II MH alpha sub-unit appeared unchanged at the protein level after 48 hours in the presence of live *Achlya* or *Saprolegnia*, as well as after 10 days in the presence of heat-killed *Achlya* preparations (Fig. 5-7). Subsequent transcript analysis revealed that, while heat-killed *Achlya* does induce the cyclooxygenase gene, COX-2, after 48 hours, the pro-inflammatory gene TNFα as well as the class II MH sub-units (CIIα & CIIβ; Fig. 5-8) and its associated chaperone molecule, S25-7 demonstrate little to no significant change with respect to the control (Fig. 5-8).

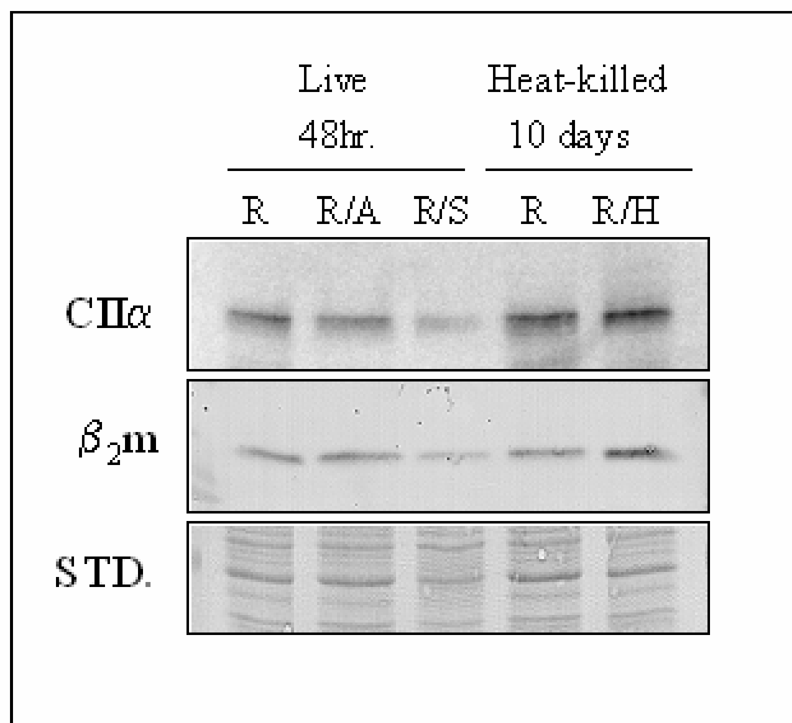

Figure 5-7. MH protein expression in the presence of *Achlya* & *Saprolegnia*.

Previously developed affinity-purified polyclonal antibodies, raised against recombinant forms of trout MH sub-units, were employed against RTS11 lysates following treatments indicated above. **Lane R** is lysate of RTS11 grown alone. **R/A & R/S** are lysates of RTS11 grown for 48 hr. in the presence of *Achlya* and *Saprolegnia*, respectively. **R** & **R/H** are lysates from 10 day cultures of RTS11 grown alone or in the presence of heat-killed *Achlya* inoculum, respectively. Ponceau S staining **(STD)** of membrane, prior to blocking and subsequent probing, indicates protein transfer and serves as a measure of equal loading among lysates.

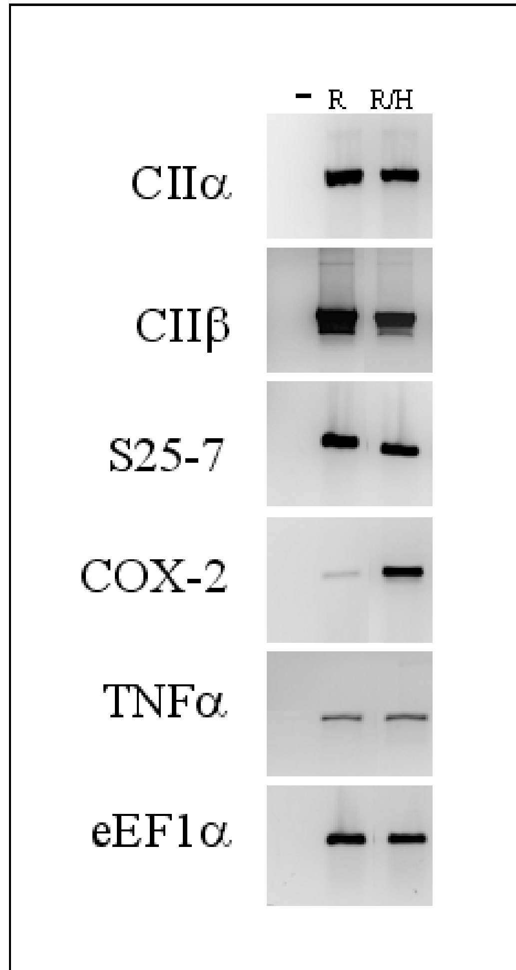

Figure 5-8. RTS11 gene expression in the presence of heat-killed *Achlya.*

Target cDNAs are indicated on the left from 48 hr. cultures of RTS11 alone (R) or grown the presence of heat-killed *Achlya* mycelium and spores **(R+H)**. PCR reactions included water in place of cDNA template to serve as negative controls, indicated with a *minus sign* **(-)**. .cDNA template was derived from RTS11 grown for equal time in the presence of heat-killed *Achlya* mycelium and spore preparations. Equal amounts of total RNA, determined by absorbance at 260nm, were used in cDNA synthesis reactions. Band intensity serves as a measure of original transcript abundance relative to the internal standard, eEF1α as previously described by Hansen & Strassburger, 2000.

5.4. DISCUSSION

5.4.1. Cellular effects of fungal-macrophage co-culture

The rainbow trout monocyte/macrophage cell line, RTS11, demonstrated cell adhesion to spores and mycelia of the Saprolegniales water molds: *Achlya bisexualis* and *Saprolegnia parasitica* (Fig. 5-1). Heat-killing the fungus did not affect cell adhesion, suggesting a heat tolerant factor mediates this response and did not require active fungal participation (Fig. 5-3). Preliminary data suggests that adhesion is not due to the presence of cellulose or simply to a physical substrate and is cell-type specific, as adhesion was not demonstrated by the trout gonadal fibroblast cell line, RTG-2.

Macrophage adherence appeared to occur at non-uniform sites along fungal hyphae, as some regions were devoid of macrophage attachment. These areas of adherence may contain lipid-like fungal components, consisting of diacetylated ureas, which have been previously isolated from yeasts and shown to induce neutrophil adherence and degranulation (Schroder *et al.* 2002). Some fungal groups are known to produce secondary metabolites that in mammals are immunosuppressant and cytotoxic (Watanabe et al., 2003). One example is gliotoxin, which causes apoptosis in macrophages, including RTS11 (DeWitte-Orr & Bols, 2005). As RTS11 viability was unchanged in co-cultures with the fungi, the fungal species under study did not appear to produce toxins, which supports earlier *in vivo* observations (Neish, 1977). Unlike a previous *s*tudy using *Aphanomyces* as the pathogen (Miles *et al.* 2000), the trout macrophage cell line did not demonstrate any evidence of phagocytosis of *Achlya* spores and is likely due to the difference in spore size between these species. Encysted zoospores of *Aphanomyces* are ≤ 5µM in diameter, while *Achlya* and *Saprolegnia* spores range from 10-20µM, (Dick, 2001) and likely exceed the phagocytic capabilities of the trout macrophage, which similarly range in size from 7- 15µM in diameter (Zelikoff & Enane, 1991).

At higher cell densities, RTS11 demonstrated HA in the presence of both live and heat-killed *Achlya* (Fig. 5-5). This aggregation was replicable as it also developed in freshly plated cells using conditioned media from either *Achlya* grown in media alone or

in co-culture with RTS11 (Fig. 5-5). The fact that homotypic aggregation was produced most prominently by conditioned media from co-culture seemed to suggest a synergistic effect between potential factors released by both the fungus and RTS11. Also, the HA induced by *Achlya* was distinct from that induced in RTS11 by double stranded RNA, which appeared not to be mediated by the release of factors into the medium (DeWitte *et al*., in press). HA is an inflammatory response demonstrated among macrophage, neutrophils and eosinophils to various foreign bodies, including wool, dust, bacterial lipopolysaccharide (LPS), phorbol myristate acetate (PMA) and leukotrienes (Teixeira *et al*. 1995). In mammals, HA induction has been shown to be divalent cation-dependent, implicating the involvement of integrins. (Teixeira *et al*. 1995). RTS11, when in the presence of *Achlya*, demonstrated moderate up-regulation of calreticulin (Fig. 5-6), which is the major calcium-binding protein of the endoplasmic reticulum and suggestive of a calcium-mediated response. Similar to the mechanism of LPS and PMA induction, HA demonstrated in this study, by RTS11 in the presence of these fungal species, could be induced synergistically through the conversion of fungal arachidonic acid to leukotriene and through the CD14-linked toll-like receptor 2, which recognizes yeast components (Underhill *et al.* 1999), or a Dectin-1-like receptor, specific for fungal 1,3-β-glucans (Yoshitomi, 2005). In a previous study, an undefined macrophage surface receptor antibody, named anti-aggregatin, induced calcium-dependent HA in fish leukocytes while inhibiting respiratory burst (Mulero *et al*. 2002). *Saprolegnia* and *Achlya* components may act as a ligand to one of these receptors.

5.4.2. Effects of fungal co-culture on trout macrophage gene expression

In addition to cell adherence and aggregation, RTS11 demonstrated alterations on gene expression in the presence of *Achlya* (Fig. 5-6). Cytokines involved in the inflammatory response, IL-1β and TNFα, were strongly up-regulated after 48 hours, as previously described in mice and rats, where fungal components were shown to up-regulate both IL-1β and TNFα (Bhandari & Sharma, 2002; Yike *et al*. 2005). RTS11 has been previously shown to up-regulate IL-1β along with TGFβ and COX-2 in response to bacterial LPS (Brubacher *et al*. 2000). COX-2 was also strongly induced in RTS11 when in the presence of either live or heat-killed mold. Cyclooxygenase (COX) converts arachidonic acid to prostaglandin as part of inflammatory and immunological responses

(reviewed by Cha *et al.* 2006). *Saprolegnia* is known to produce significant amounts of arachidonic acid (Gellerman & Schlenk, 1979). Whether COX-2 induction in RTS11 is simply a general inflammatory response to the growing fungus or a response to the presence of excess arachodonic acid remains unclear; however, induction and aggregation in the presence of heat-killed mycelium suggests the latter (Fig. 5-8). Interleukin-8 (IL-8), which is involved in chemotaxis of neutrophils and granulocytes, was moderately up-regulated in the presence of the mold and may represent a means for recruiting other cell types. This is particularly interesting as the CC chemokines, which generally attract monocytes, lymphocytes, basophils, and eosinophils (Laing & Secombes, 1999) showed no detectable induction in the presence of the mold. CD9, shown to be involved in cell-to-cell adhesion in mammals, appeared slightly down-regulated suggesting an alterative mode of cell adhesion.

As an extracellular pathogen, *Achlya* failed to elicit change in expression of the inducible antiviral proteins Mx 2&3 as well as the class I MH receptor, typically reserved for endogenous (viral) antigen recognition. It should be noted that class I MH can serve as an alternative means of exogenous antigen recognition, especially among phagocytic macrophage (Bevan, 1987); however, the inability to phagocytose these larger fungal spores reduces the possibility for such cross-priming, a response perhaps reserved for smaller spores such as those of *Aphanomyces*. The class I chaperone, calreticulin showed moderate up-regulation, which may highlight its dual function as the primary means of cellular calcium retention, a requirement for integrin-mediated cell adhesion as discussed above. Following the lack of class I mediated response, STAT-1, a signaling component of various extracellular ligands and previously shown to be strongly up-regulated in trout following acute exposure to viral infection (Hansen & La Patra, 2002), appeared unchanged in the presence of the mold.

In addition to respiratory burst, macrophage employ another free-radical pathogen attack system through inducible nitric oxide synthase (iNOS). Reactive intermediates produced by iNOS are known to be an important response to invading pathogens in mammals (Nathan & Hibbs, 1991) and fish (Laing *et al.*1999), however this gene was not up-regulated in the presence of the mold and may require the presence of gamma interferon (IFN γ) or be actively suppressed by the fungus. Interestingly, *Candida*

albicans has been previously shown to suppress macrophage iNOS expression while stimulating IL-1β in mice (Chinen *et al*. 1999), similar to what was demonstrated in the present study. It has previously been shown that in the presence of *Aphanomyces*, macrophage activity and respiratory burst are significantly reduced, which further suggests the possibility of fungal interference (Miles *et al*. 2000), perhaps through a similar mechanism as anti-aggregatin, described previously (Mulero *et al*. 2001).

The most notable effect was on the genes encoding the class II MH antigen presentation machinery, which includes genes encoding the class II receptor and its chaperone, the invariant chain, which all appeared down-regulated in the presence of the fungus (Fig. 5-6). A glycoprotein, previously isolated from the fungus *Paracoccidioides,* has been shown to lead to a similar down-regulation of class II MH and adhesion in immature mammalian dendritic cells, suggesting a role in immunosuppression during infection (Ferreira *et al*. 2004). Such a response is not typical among all fungi as many studies demonstrate up-regulation of class II MH in macrophage (Rodriquez-Galán *et al*. 2002; Bacci *et al*. 2002) however *Saprolegnia parasitica* has been shown to produce novel compounds including arachidonic acid (reviewed by Noverr *et al*. 2003), the direct precursor of eicosanoids such as prostaglandins, leukotrienes and thromboxanes, which are known to down-regulate macrophage activity in mammals (Sadick, 1992) and fish (Secombes *et al.* 1996). Reduction in RTS11 class II MH expression appeared to be less dramatic at the protein steady-state level (Fig. 5-7), suggesting the requirement for longer exposures to the live fungus however a subsequent 10 day exposure to heat-killed mycelium failed to elicit the transcript down-regulation demonstrated with live preparations implying the need for active participation by the fungus (Fig. 5-7). Active down-regulation of class II MH by these molds may serve as a form of immune evasion as the class II MH receptor plays a crucial role in the recognition of exogenous antigens including bacteria and fungi.

5.5. CONCLUSIONS

Based upon these data, the rainbow trout monocyte/macrophage cell line, RTS11 appears to activate and adhere specifically in response to both live and heat-killed water mold mycelium and their culture filtrates. Though gene transcript levels clearly demonstrate a cellular response through significant cytokine and cyclooxygenase up-regulation as well as cellular aggregation, the ability of the macrophage cell line to inhibit mold growth was not evident. In addition, this report indicates that the live fungus may suppress fungal antigen recognition through class II MH down-regulation. Miles and others demonstrated that low-temperatures lead to an increase in macrophage activity and respiratory burst suggesting an increase in macrophage dependence at low temperatures. Recent data, however has shown that rainbow trout, when subjected to low temperatures ($2^{o}C$), demonstrate a dramatic reduction of leukocyte class II MH receptor expression (Nath *et al*. 2006). This reduction in exogenous antigen recognition machinery, along with reduced respiratory burst and iNOS activity, would greatly hinder any macrophage-mediated response to an invading fungal pathogen and may explain, at least in part, the increased frequency of infection by these molds following dramatic decreases in water temperature (Bly *et al.* 1992; Bly *et al.* 1993). Low temperatures may therefore provide a means for fungal invasion, while active fungal suppression of class II and its associated molecules, shown here, may facilitate immune evasion following a return to warmer water temperatures.

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Chapter 6

Fishing for a Mate:

Salmonid Major Histocompatibility Expression in Reproductive Tissues

ABSTRACT

The class I & II Major Histocompatibility (MH) receptors serve a critical role in self/non-self recognition through the presentation of peptide antigen to circulating T lymphocytes. MH heterozygosity, and therefore the ability to recognize a diverse repertoire of foreign antigen, can provide inheritable benefits to offspring. A growing amount of evidence indicates the presence of MH-based mate selection in mammals and fish; however little is known of the mechanism through which MH genotype is scrutinized. Here, through the development of polyclonal antibodies towards rainbow trout MH receptor sub-units, we report the expression of these immune molecules in the reproductive tissues and fluids of rainbow trout and the presence of a secreted form of the MH I receptor in seminal fluid. Western blot analysis revealed that trout MH I exists primarily as a 45kD glycoprotein in most tissues; however a smaller 42kD form is also present in most individuals and is likely due to allelic differences in amino acid composition. These forms of MH I, along with beta-2-microglobulin are present in both ovarian and sperm lysates. Despite being restricted to antigen presenting cells, MH II sub-unit expression was also detected in trout ovary and spermatozoa, as previously described in mice. A soluble 34kD, non-glycosylated form of MH I was found in both blood serum and seminal fluid and had an isolelectric point similar to that predicted of a truncated soluble form. How this data relates to MH-based mate selection is discussed.

6.1. INTRODUCTION

The Major Histocompatibility Complex (MHC) is a unique region of gDNA containing genes involved in antigen presentation and found on a single chromosome in tetrapods. Named for its role in transplant rejection, the MHC is generally described as comprising three distinct genetic regions, designated as class I, II & III and encoding numerous immune molecules, as well as heat shock proteins and proteasomal sub-units (Klein, 1986). Most notable however, are the two cell-surface molecules that bear its name, the class I & II MHC receptors. The MHC I receptor is a cell-surface heterodimer comprising a membrane-bound 45kD heavy "alpha" chain and a 12kD, non-covalently associated light chain, beta-$_2$-microgloblulin (β_2m). Though not encoded within the MHC region, the β2m light chain is critical to the cell-surface stability of this receptor (Roct *et al.* 1991; Vitiello *et al.* 1990). As a member of the Immunoglobulin (Ig) superfamily, the class I alpha chain is a glycoprotein containing three Ig domains, designated alpha 1, 2 & 3. The alpha 1 & 2 domains are highly polymorphic and form the peptide binding groove suitable for binding a diverse set of peptide antigens. This receptor is expressed by nearly all nucleated cells where, in concert with antigen processing, it presents peptide antigens to circulating T lymphocytes. Typically these peptides are the products of proteasomal degradation, serving as a snapshot of cellular processing and thereby providing a means of self/non-self discrimination through the recognition of viral, tumour and transplant peptides. The MHC II receptor is also a heterodimeric molecule comprised of a membrane-bound alpha and beta chain. Having molecular masses of roughly 30kD (Blair *et al.* 1995), these two sub-units, with their highly polymorphic Ig domains, form a peptide binding groove similar to that of the class I receptor. Peptides bound to this receptor are of exogenous origin, generated through the lysosomal pathway, and therefore typically represent bacterial, fungal and parasitic antigens. Unlike the ubiquitous class I receptor, the MHC II receptor has limited expression and is typically restricted to cells involved in extracellular antigen presentation such as: B cells, neutrophils, macrophages and dendritic cells. Together the class I and class II MHC receptors serve in the recognition of foreign peptide antigens and are critical to both innate and adaptive immune responses.

Due to their role in pathogen recognition, the MHC and its components have been extensively studied in mammals; however, only recently have functional studies included other vertebrates. Unlike all tetrapods, where MHC is located on a single chromosome, the teleost homologues of these genes have been found to be located on separate chromosomes (Phillips *et al.* 2003) and should therefore be referred to as simply MH genes (MHg; Shand & Dixon, 2000). Despite this dramatic difference in genomic organization, the teleost MHC homologues are generally believed to serve a similar function in fish as they do in humans and all other jawed vertebrates (Vallejo *et al.* 1992).

Due to their polymorphic nature and the selective pressures of disease, MHC genes and receptors can provide a heritable defence against numerous recurrent pathogens. Intuitively, individuals possessing heterozygous MHC genes should express a more diverse set of MHC alleles and consequently a similarly diverse antigen binding repertoire capable of the recognition of a variety of pathogens. Like many characteristics, MHC genes can therefore provide offspring with a valuable resource for survival and are considered selectable genes for fitness (Neff & Pitcher, 2005). Several studies have investigated the role MHC genes may play in mate selection. In humans, one study demonstrated odour preference by females towards potential suitors who carried MHC genes dissimilar to their own (Wedekind *et al.* 1995). Odour preference was found to be towards mates that would provide the most diverse set of MHC genes to subsequent offspring. Mice share a similar odour preference and olfaction studies have demonstrated that mice can discriminate a single point mutation in MHC class I genes of their mates (Penn & Potts, 1998), while further studies have shown evidence of post-copulatory sexual selection, selected abortion and non-random fertilization correlating with the MHC genotype of mates (reviewed by Wedekind *et al.* 1996). The role of MHC genes in post-copulatory sexual selection has been discussed previously (Birkhead & Pizzari, 2002) and the presence of MHC antigens on the surface of spermatozoa, which could facilitate female discrimination of the MHC composition of their mates, has been a matter of some controversy, due to contradicting reports of MHC gene expression in various stages of germ cells. To date, MHC gene expression has been clearly identified in the spermatozoa of both humans (Martin-Villa *et al.* 1999) and mice (Hotta *et al.* 2000) and appears to be transcriptionally regulated through cytokines (Hotta *et al.* 2000) and

hormones (Martin-Villa *et al.* 1999), which may explain the history of contradictory reports. In humans, spermatozoan MHC gene expression has been shown to be cyclic and negatively correlated with inhibin levels, which the authors suggested may play a role in regulating male fertility (Martin-Villa *et al.* 1999). In fish, which have since become a common model for studying mate selection, three-spined Stickelback (*Gasterosteus aculeatus*) females demonstrate odour preference for mates expressing the most different class II MH alleles (Reusch *et al.* 2001), while in Atlantic salmon, (*Salmo salar*) which unlike Sticklebacks have only a single class II locus, evidence shows that females select mates based upon diversity within the peptide binding region of their MHC receptors (Landry *et al.* 2000).

Clearly fish, like mice, have the capacity to detect minor differences in their mate's MH genotype; however little is known regarding the mechanism behind this MH-based mate scrutinization. Recent evidence has shown that peptide ligands can be detected by olfactory neurons in sticklebacks and may provide an indirect means of MH discrimination (Milinski *et al.* 2005). In this study, through the development of polyclonal antibodies towards rainbow trout MH receptor sub-units, we report the presence of these immune molecules in the reproductive tissues and fluids of rainbow trout and that a truncated, soluble form of MH I is present in seminal fluid. How these receptors may serve in mate selection is discussed.

6.2. MATERIALS AND METHODS

6.2.1. Fish

Rainbow trout, (*Oncorhynchus mykiss*) were obtained from Mimosa Springs Trout Farm, Guelph, Ontario and held in well-water flow-through tanks at the University of Waterloo on a daily diet of pellet ration. For antibody testing, specimens of Atlantic salmon, (*Salmo salar*) were kindly provided by Dr. P. Woo at the University of Guelph, while those of the common carp, (*Cyprinus carpio*) and Arctic char, (*Salvelinus alpinus*) were kindly provided by Dr. D. Barton and Dr. M. Power from the University of Waterloo. For tissue collection, trout weighing approximately 450 grams were sacrificed using a lethal dose of ethyl 3 aminobenzoate methanesulfonate salt (MS222; Sigma)

followed by caudal vein exsanguination. Tissues were extracted and flash frozen in liquid nitrogen prior to storage at –80°C. For serum isolation, caudal blood was allowed to clot at room temperature for 2 hr. then chilled on ice for 10 min. prior to centrifugation at 3 000 g for 10 min. at 4°C. The serum was then transferred to a fresh tube containing sodium azide (Sigma, St. Louis, MO) to a final concentration of 0.02%. Rainbow trout sperm, as well as seminal & ovarian fluids were kindly provided by Michael Burke, from the Alma Aquaculture Research Station at the University of Guelph. Reproductive cells were pelleted by centrifugation at 1 000 g for 5 min., then washed in 1X PBS. The supernatant fluids were then filtered through 0.2 μM syringe filter to remove any remaining cells prior to analysis. An aliquot of sperm cell preparation was examined microsocpically to ensure a homogeneous suspension (not shown).

6.2.2. Production of recombinant trout MH I

All nucleotide sequence analysis, including oligonucleotide design, deduced amino acid sequence and isoelectric points (pI) were performed using GeneRunner version 3.05 (Hastings Software, Inc.). Oligonucleotides were designed to amplify the extracellular coding region of rainbow trout alpha chain using the published classical trout MH I cDNA allele (UCA-C32; PubMed accession # U55380; bp# 115-981). 5' GC clamps preceding *Bam*HI and *Bgl* II restriction sites were included in the sense and antisense primers, respectively to facilitate enzymatic digestion and subsequent ligation into the prokaryotic expression vector, pRSET A (Invitrogen). The antisense primer also included a stop codon to exclude translation of vector sequence. PCR primers and cycling parameters were performed as follows using a rainbow trout thymus cDNA library as a template: RTUCAF1sense (5'-GCGCGGATCCGTGACTCACTCCCTGAAG-3'; bp#115-132), RTUCACPrevBgl2 antisense (5'-CGCCTCTAGACTAATTTGAACCCCT-3'; bp#981-969), 95°C for 5 min., 30 cycles (95°C for 45s, 48°C for 45s, 72°C for 1m 30s), 72°C for 10 min. The resulting amplicon was ligated into pGEM T easy (Promega, Madison, USA) and sequenced by dideoxy terminating method on a long-read tower sequencer (Visible Genetics). Following sequence scrutiny, the amplicon was excised using a *Bam*HI and *Bgl*II (MBI Fermentas) double-digest at 37°C overnight (Invitrogen) and purified by 1% agarose gel electrophoresis extraction, as described above.

Similarly, the pRSET A expression vector was prepared for ligation using *Bam*HI and *Bgl*II digestion followed by a 30 min incubation with calf intestinal alkaline phosphatase according to manufacturer's instruction (CIAP; Promega). The purified MH I coding sequence was ligated into the pRSET A backbone by overnight incubation with T4 ligase (Promega) at 4^oC. Following sequence verification, the pRSET A-MH I fusion vector was transformed into *E. coli* BL21 (DE3) competent cells (Invitrogen). Transformed cultures were screened from a Luria-Bertoni (LB) broth containing 100 μg/mL ampicillin at 37^oC with shaking to an OD600=0.5 prior to induction using 1 mM IPTG. Cultures were pelleted after 3h and lyzed using denaturing conditions (8M urea) according to manufacturer's instructions (Invitrogen). Recombinant trout MH I, which was a fusion peptide containing an N-terminal 6XHis tag and AntiXpress epitope, was purified using Ni-NTA agarose according to manufacturer's instructions (Invitrogen) and verified by western blotting using AntiXpress antibody (Invitrogen).

6.2.3. Development of Trout MH I antisera

Pooled elutions of purified recombinant trout MH I were dialyzed out of denaturing buffer using a gradual four-step-wise decrease in urea concentration into 1X PBS. A total of 3 mg of recombinant protein was injected intramuscularly (*i.m*) into 2 New Zealand white rabbits emulsified 1:1 in Freund's complete adjuvant (FCA) followed by 3 subsequent boosts at 3 week intervals using Freund's incomplete adjuvant (FIA). Ear bleeds were regularly performed to analyze rabbit antibody titre prior to exsanquination. Subsequent polyclonal antibodies were purified from the rabbit serum using Sulfolink resin charged with 3 mg of purified recombinant trout MH I according to manufacturer's instructions (Pierce).

6.2.4. Western blot analysis

Approximately 200 mg of wet tissue was thawed and sonicated in 500 uL of lysis buffer (1% NP-40, 150mM NaCl, 10mM Tris; pH 7.4 supplemented with 2mM phenymethylsulphonyl fluoride (PMSF; Sigma) and protease inhibitor cocktail (Sigma)). Crude tissue lysates were then cleared by centrifugation at 4^oC for 10 min at 21,000 g. Cleared lysates, as well as filtered fluids, were analysed by Bradford method to determine and equalize total protein concentration among samples. Approximately 100 μg of total protein was separated by 15% SDS-PAGE and electroblotted to nitrocellulose membrane.

Total protein transfer was determined using 0.2% Ponceau S (Fisher) in 5% acetic acid and recorded using a flatbed scanner then washed and blocked for 1 h in TBS-Tween containing 5% skim milk powder. Blots were probed for 1 h at room temperature using affinity-purified rabbit antisera then washed three times in TBS-Tween for 5 min each, prior to secondary probing using goat anti-rabbit alkaline phosphatase (Sigma) at 1: 30 000 for 45 min. Reactive banding was detected using NBT/BCIP and digitally recorded on a flatbed scanner. Equal loading was determined by relative band detection following Ponceau staining.

6.2.5. De-glycosylation

For *in vitro* de-glycosylation, trout gill lysates were treated as previously described (Dijkstra *et al.* 2003). Approximately 500 µgs of total protein was concentrated using a 3 000 MWCO spin column (Millipore) according to manufacturer's instructions. Lysate proteins were resuspended in 200 µL of sterile distilled water containing 0.5% SDS, 0.1% β-mercaptoethanol and boiled for 10 minutes. The cooled lysate was treated overnight at 37°C with 2500 U of Endo H (New England BioLabs) in a 50 mM sodium citrate buffer; pH 5.5 followed by an additional overnight incubation at 37°C following the addition of 20 U N-glycosidase F (Roche) in 50 mM sodium phosphate; pH 7.5 containing 1% NP-40. A mock treatment (-), containing equivalent volumes of purified water in place of enzymes, served as a reaction control. Reaction volumes equivalent to 50 µgs of original total protein were separated on 15% SDS-PAGE and blotted onto nitrocellulose membrane for subsequent probing using rabbit anti-trout MH antisera, as described above.

6.2.6. Purification of soluble MH I

Soluble trout MH I was purified using a two-step method. Briefly, affinity-purified rabbit serum, raised against recombinant rainbow trout MH I, was coupled to Sulfolink resin (Pierce) according to manufacturer's instructions and employed to isolate soluble MH I from neat trout serum. MH I was then immunoprecipitated by mixing peak elutions with affinity-purified MH I antisera at 4°C for 2 hr. in the presence of protein G Sephadex (Roche). The complex was then precipitated through centrifugation at 1 000g for 2 min., followed by 5 washes using 1X PBS.

The washed resin was boiled in modified Laemmli buffer for 5 min. and the resulting supernatant was employed for two-dimensional SDS-PAGE and western blot analysis.

6.3. RESULTS

6.3.1. Development of polyclonal antisera to recombinant trout MH I heavy chain

Oligonucleotides designed to amplify the extracellular domain of rainbow trout MH I heavy chain produced an 867bp PCR amplicon having a deduced amino acid sequence exactly matching the extracellular portion of UCA 0401 (PubMed accession#: AY523662), a previously published classical rainbow trout MH I heavy chain allele belonging to the *Onmy*-IB region (Shina *et al.* 2005; Dijkstra *et al.* 2006). Competent *E. coli* BL21(DE3), transformed with the pRSET A-MH I fusion vector, expressed a ~36kD inducible protein, which was recognized by the AntiXpress antibody (not shown; Invitrogen) and demonstrated nickel affinity. Affinity-purified rabbit polyclonal antisera, raised against this purified product, reacted strongly to a ~45kD polypeptide in gill lysates of both rainbow trout & Atlantic salmon, but not to those of the common carp or Arctic char (Fig. 6-1). Subsequent western blot analyses revealed fish to fish variability among rainbow trout as a second lower band, having a relative molecular mass of ~42kD, was also detected in many individuals (Fig. 6-2). Tissue distribution assays revealed that these proteins were broadly expressed, with highest relative expression in immune tissues, including: spleen, head-kidney, intestine and gill, while lowest relative expression was detected in muscle, brain and liver, which is similar to previous northern blot analysis of other classical trout MH I alleles (Hansen *et al.* 1999; Dijkstra *et al.* 2003). In addition to the typical 45 & 42kD proteins detected in most tissues, the affinity-purified antisera also demonstrated strong rectivity towards a polypeptide with a relative molecular mass of approximately 35kD in several tissues and erythrocytes (Fig. 6-2). Each of these three reactive proteins demonstrated mobility shift following enzymatic de-glycosylation indicating that tissue MH I proteins in trout are subject to N-glycosylation (Fig. 6-3).

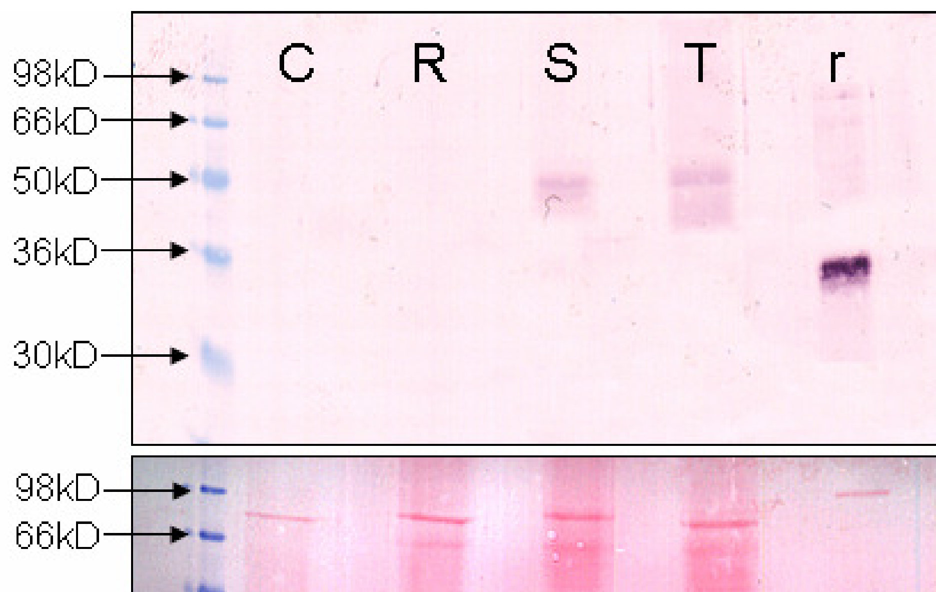

Figure 6-1. Antiserum specificity towards various fish gill lysates.

Western blot analysis of affinity-purified rabbit anti-trout MH I heavy chain antiserum to gill lysates from the common carp (C), Arctic char (R), Atlantic salmon, (S) and rainbow trout (T). The purified recombinant trout MH I fragment used for raising the antiserum (r) serves as a positive control. Relative molecular weight standards are indicated on the left (SeeBlue; Invitrogen). Lysates were equalized for protein content based upon Bradford analysis prior to loading, while Ponceau staining (lower panel) indicates total protein transfer prior to membrane blocking and probing with the antiserum.

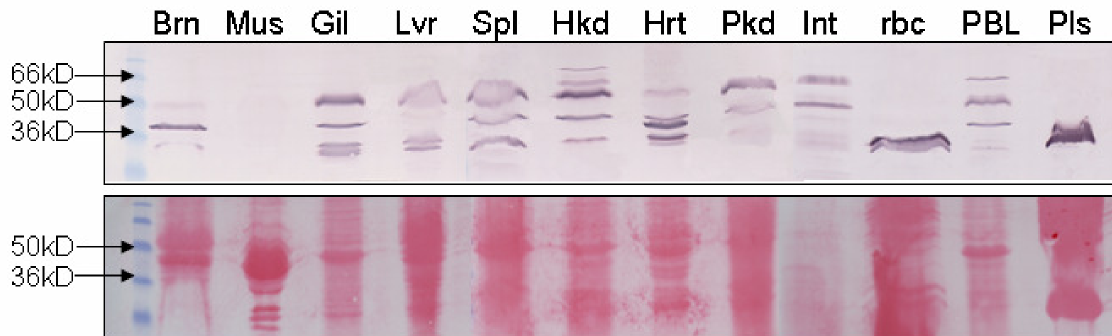

Figure 6-2. Western tissue distribution assay for anti-trout MH I reactivity.

Western blot analysis demonstrated MH I reactivity in most rainbow trout tissues and cells tested. All tissues were obtained from one individual and include brain (Brn), dorsal skeletal muscle (Mus), gill (Gil), liver (Lvr), spleen (Spl), head-kidney (Hkd), heart (Hrt), posterior kidney (Pkd), intestine (Int), red blood cells (rbc), peripheral blood leukocytes (PBL) and caudal blood plasma (Pls). Ponceau staining (lower panel) indicates total protein transfer prior to membrane blocking and probing with the antiserum.

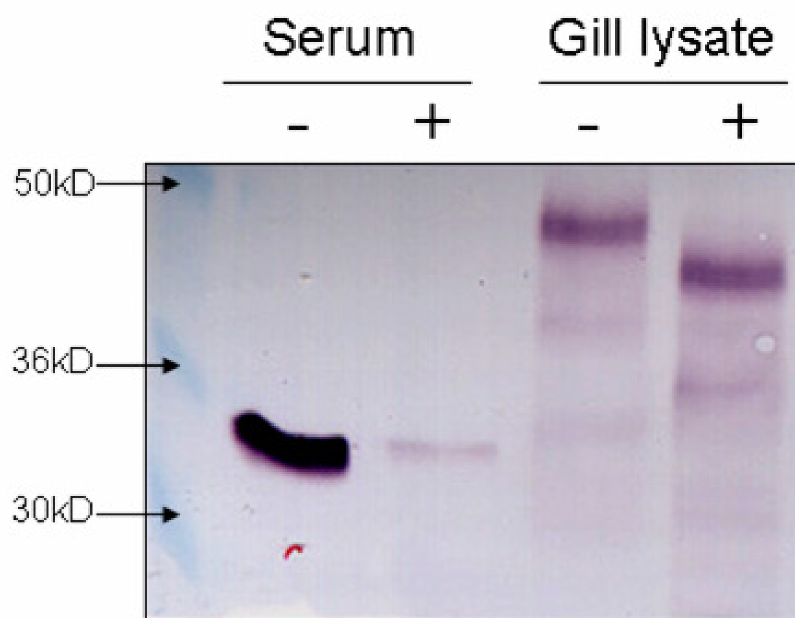

Figure 6-3. Differential N-glycosylation of trout MH I.

Western blot analysis of MH I reactivity in rainbow trout serum and gill lysate following *in vitro* de-glycosylation (+). Mock reactions (-) contained no enzyme. Mobility shift in gill indicates MH I is subject to N-glycosylation, while serum bands did not shift their mobility indicating no N-glycosylation of these polypeptides.

6.3.2. MH I expression among trout reproductive tissues & fluids

MH I expression was also detected in reproductive tissues of both male and female specimens, as sperm and ovarian lysates contained polypeptides of similar mass as those described in the immune tissues above (Fig. 6-4). Western blot analysis using antisera, recognizing trout beta-$_2$-microglobulin (β_2m), the light chain component of the MH class I receptor (previously described in Kales *et al*. 2006), also revealed relatively high expression of this sub-unit in ovary, ovarian fluid and seminal fluid and only limited detectable expression in sperm lysate (Fig. 6-4). In addition to detecting the forms of MH class I shared among most tissue lysates, affinity-purified antisera also demonstrated strong reactivity towards a polypeptide having a relative molecular mass of approximately 34kD, in trout ovarian lysate, seminal plasma (Fig. 6-4) and blood serum (Fig's. 6-2 and 6-3). The molecular mass of this MH class I polypeptide is slightly lower than those detected in other tissues and less than any known trout MH I allele. Unlike the comparable 35kD polypeptide detected above in several tissues, this polypeptide did not display a mobility shift following N-type de-glycosylation (Serum; Fig. 6-3). The larger MH I forms, found in most of the tissue lysates, were not detected in the seminal fluid or blood plasma, leaving this soluble, non-glycosylated 34kD polypeptide as the sole detectable MH I reactive polypeptide in these fluids. To assess whether or not these smaller than usual MH class I polypeptides detected in reproductive fluids were simply the product of cell lysis, antisera raised against rainbow trout calreticulin, an ER resident chaperone, (previously described in Kales *et al.* 2006), was used to probe the same membrane. Calreticulin was only found in cell lysates and not detected in ovarian and seminal fluids (CRT; Fig. 6-4). Following a two-step purification protocol, western blot analysis of a two-dimensional gel electrophoresis separation of serum MH I revealed that this 34kD polypeptide has a differential relative isoelectric point (pI) ranging from approximately pH5 to 6 (Fig. 6-5), as multiple points of MH class I antiserum reactivity were detected (Fig. 6-5).

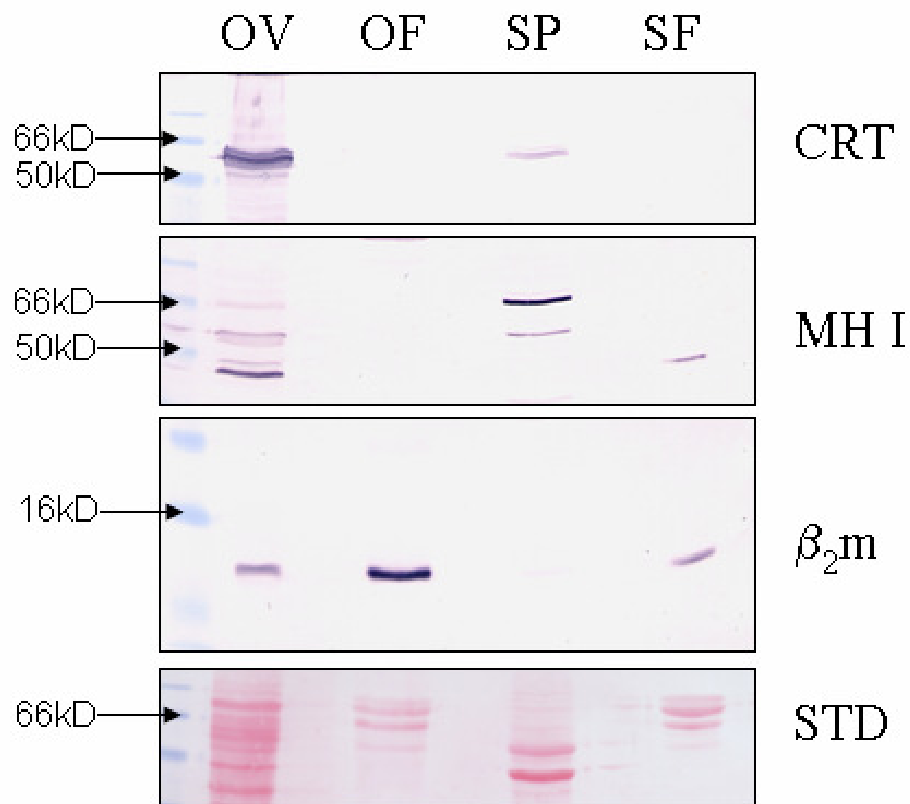

Figure 6-4. MH I protein expression in trout reproductive tissues and their fluids.

Western blot analysis of trout lysates for the detection of calreticulin (CRT), MH class I and beta-2-microglobulin (β2m). Lanes contain trout ovary (OV) and sperm (SP) lysates as well as ovarian fluid (OF) and seminal fluid (SF). Ponceau staining (STD) indicates total protein transfer between lanes prior to membrane blocking and probing with the appropriate antiserum.

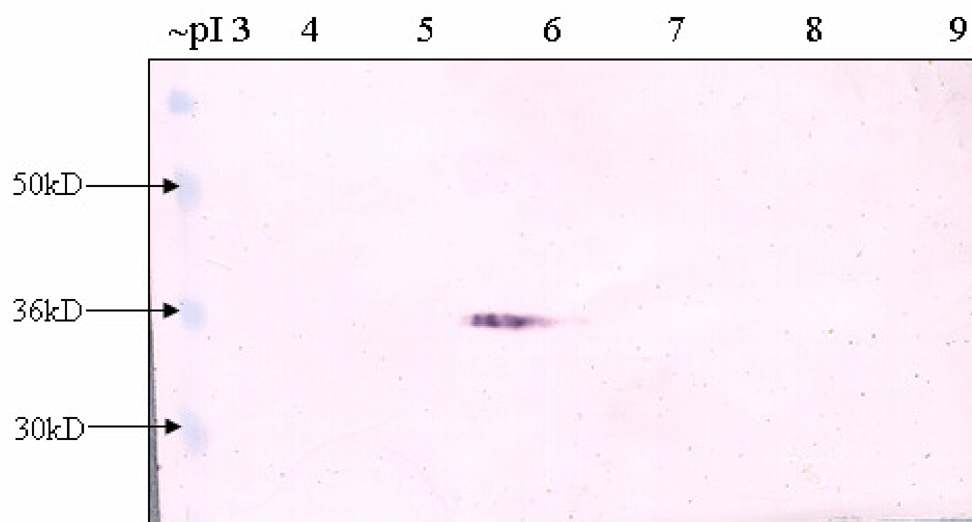

Figure 6-5. Two-dimensional western blot analysis of purified serum MH I.

Purified trout serum protein was separated by 2D-gel electrophoresis and transferred to membrane for subsequent western blot analysis with MH class I specific antiserum. Reactivity was detected at multiple points between pI 5 & 6, at a molecular mass of 34kD.

6.3.3. MH II expression in trout sperm and ovary

Though no soluble forms of MH II were found in the trout reproductive fluids, western blot analysis of rainbow trout sperm and ovarian lysates revealed detectable levels of the class II MH alpha sub-unit using an affinity-purified antiserum previously developed in our laboratory (Nath *et al.* 2006). This antiserum, raised against a recombinant form of *Onmy*-DAA, reacted strongly to a polypeptide having a relative molecular mass of 35kD (MH II α; Fig. 6-6), the appropriate size for this N-glycosylated protein (Nath *et al.* 2006). Expression of the class II MH beta sub-unit, similarly raised against a recombinant *Onmy*-DBA, was also detectable in ovary (MH II β; Fig. 6-6); however sperm lysate demonstrated only limited levels of detectable MH class II beta protein following increased blot exposure time (data not shown).

6.4. DISCUSSION

Class I & II MHC play a pivotal role in self/non-self recognition in mammals. Homologues of these genes and those encoding several of their associated molecules have now been identified throughout the jawed vertebrate lineage, where they are believed to serve a similar role in immune recognition. Due to the genetic fitness these polymorphic genes can convey upon offspring, numerous reports have investigated their involvement in mate selection; however little is known of the mechanism for both pre and post-copulatory mate choice in fish or mammals. Through the development of polyclonal antiserum, this report demonstrates that MH I heavy chain in rainbow trout exists as a 45 & 42kD protein (Fig. 6-2). In addition, a smaller, 35kD form of this peptide exists within some tissues and cell lysates. All three of these forms demonstrated mobility shift following *in vitro* de-glycosylation (Fig. 6-3), suggesting that the 45 & 42kD forms are not the products of differential glycosylation, but rather likely represent the products of two alleles, each producing proteins of slightly different relative molecular masses due to differences in amino acid composition. Such amino acid variability is an important feature for this antigen binding receptor; however, despite its polymorphic nature, MH I maintains 75% amino acid identity among known alleles due to conserved regions, which likely facilitates antibody recognition of numerous alleles.

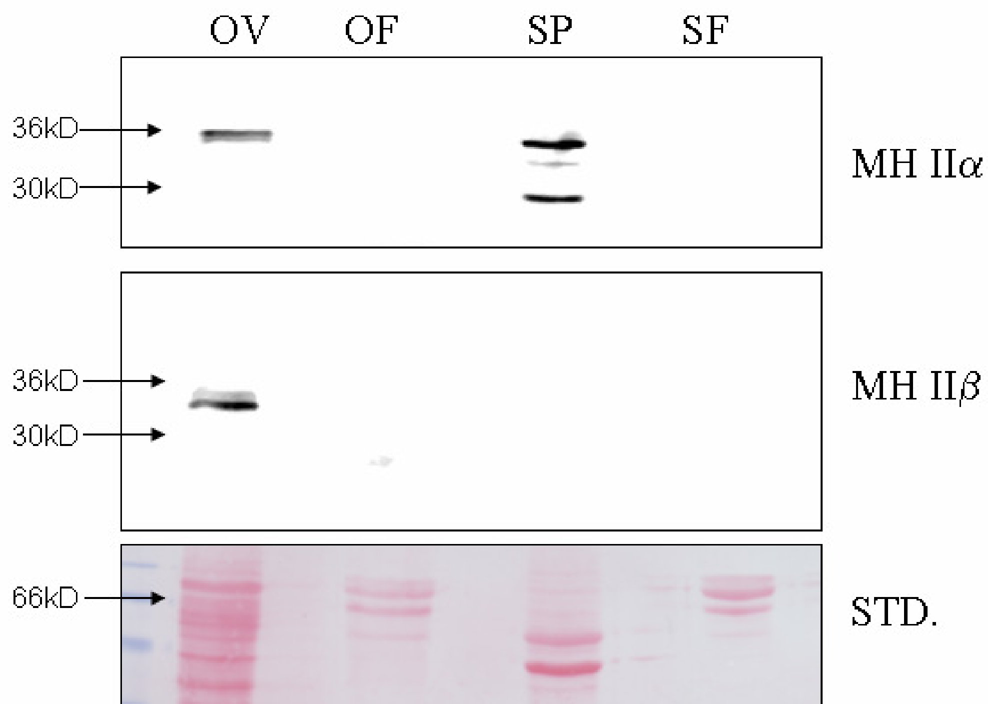

Figure 6-6. MH II expression detectable in trout sperm and ovary lysates.

Western blot analysis of MH II α and β sub-unit expression in trout ovary (OV) and sperm lysates (SP) and their corresponding fluids. Ponceau staining (STD) indicates total protein transfer prior to membrane blocking and probing with the appropriate antiserum.

The 35kD polypeptide, noted in some tissues and cells, is also subject to N-glycosylation, but is smaller than any known allele and may represent proteolytic degradation of cytoplasmic sources of the larger allelic forms, or perhaps an alternatively spliced product. Alternative splicing and premature stop insertions have been recently demonstrated in fish MH genes (Roney *et al.* 2004; Fujiki *et al.* 2005) and suggested for some trout heavy chain alleles (Dijkstra *et al.* 2006) and may be the source of this polypeptide. Interestingly, only the lower relative molecular mass MH I polypeptide was detected in trout erythrocytes. Low-level MH I specific reactivity has been previously detected in trout erythrocytes (Dijkstra *et al.* 2003). Whether this polypeptide represents the proteolytic remnants of a mature MH I produced during early erythropoiesis or is transcribed as a truncated product remains unclear.

Both of the larger forms of MH I are expressed in most tissues, with highest expression in immune type tissues (Fig. 6-2), as previously shown (Hansen *et al.* 1999; Dijkstra *et al.* 2003) as well as in lysates of ovary and sperm (Fig. 6-4). Though ubiquitous in its expression among most nucleated cell types, MH I in sperm may serve a dual purpose by facilitating MH discrimination during copulation. Evidence has demonstrated sperm selection occurs in mammals (reviewed by Anderson & Simmons, 2006) and an increased frequency of spontaneous abortions has been seen in humans sharing similar MHC haplotypes (Ober *et al.* 1993). MHC expression in human spermatozoa has been shown to correlate with inhibin levels and the authors suggested that germ cell MHC expression may serve to reduce fusion of similar MHC genotypes (Martín-Villa *et al.* 1999). Spermatozoan MH expression in trout may serve a similar function. In addition, MH I expression on spermatozoa could also serve as a source of the smaller, soluble form of MH I identified in trout seminal plasma (Fig. 6-4), and may further facilitate mate choice through pre-copulatory selection. Soluble MH, both with and without their associated light chains, have been identified in humans (Galati *et al.* 1997) and is believed to be the product of proteolytic release of the extracellular domains of the receptor from its transmembrane domain. These products range in relative molecular mass from 34 to 37kD (Demaria & Bushkin, 2000), similar to the polypeptide described in this report.

Based upon sequence data, trout MH I proteins contain several low-specificity proteolytic cleavage sites (papain and trypsin) within their connecting peptide, which could produce a fragment of the size described here. Furthermore, such a cleavage would result in a predicted change of pI for trout MH I from pH 6.2 to 5.6, which agrees with the two-dimensional western blot analysis shown here (Fig. 6-5). The multiple reactive points indicating slight differences in pI (Fig. 6-5) may represent the proteolytic products of multiple alleles, each having slight amino acid charge differences. Post-translational modifications, such as differential phosphorylation, has been previously demonstrated in mouse MHC I (Capps & Zuniga, 2000) and could provide a similar result. The soluble MH I reported here was resistant to de-glycosylation, a post-translational feature conserved at least in the sequence of nearly all trout MH I loci (Miller *et al.* 2006); however not required for recognition by lymphocytes in mammals (Parham, 1996). Non-glycosylated forms of MH I have been previously shown for cytoplasmic and some surface MH I in mammals (Cacan & Verbert, 2000; Hughes *et al.* 1997; Lee & Geraghty, 2003). Alternative splicing of classical MH I transcripts could also provide a soluble polypeptide through the excision of the exons encoding the transmembrane region, which has been previously shown for Walleye MH I (Fujiki *et al.* 2003) and platyfish MH II (Roney *et al.* 2004). Regardless of its origin, such a soluble MH I, if secreted into the surrounding environment, may serve as a means of MH-based mate discrimination, perhaps through an odour-based mechanism, as previously described for the three-spined stickleback (Reusch *et al.* 2001; Milinski *et al.* 2005).

The implication that germ cell expression of MH may serve a function other than antigen presentation may be further supported by the presence of MH II expression in both sperm and ovary lysates (Fig. 6-6). Class II alpha was clearly evident in both germ cell lysates. Reactivity towards a ~30kD polypeptide in sperm lysate was similar in size to its de-glycosylated form, previously shown in trout (Nath *et al.* 2006), and may represent differential glycosylation. Sperm lysates demonstrated lower relative levels of class II beta expression, which may reflect reduced cross-reactivity of the antiserum among alleles of this more polymorphic sub-unit. Class II MHC is typically restricted to professional antigen presenting cells, such as neutrophils and macrophage (reviewed in Glimcher & Kara, 1992).

Macrophages have been previously identified in human semen (Mori *et al.* 1990). Though not reported in trout, the presence of macrophage in trout semen samples could serve as a contaminating source of MH II; however observations of cell preparations did not reveal the presence of these significantly larger cells among our homogenous cell preparations. In addition, the low relative expression of beta-$_2$-microglobulin and calreticulin (Fig. 6-4), which are readily detected in trout macrophage lysates (Kales *et al.* in press), further precludes the presence of any significant numbers of contaminating macrophage in our sample preparations and further supports the presence of class II MH expression by trout spermatozoa. Class II MHC has been previously identified in purified murine spermatozoa, where it is believed to play a role in fertilization, which is blocked with the presence of class II MHC antigen monoclonal antibody (Mori *et al.* 1990). MH class II expression in trout sperm may serve a similar role in fertilization and provide one mechanism for class II MH scrutiny demonstrated by Atlantic salmon females in choosing their mates (Landry *et al.* 2000).

Fish, like mammals, clearly exhibit the ability to discriminate the MH genotypes of their mates. Based upon the data presented here, we believe that the soluble MH I protein detected in seminal fluid may provide one means for pre-copulatory mate selection. Following copulation, MH I and II expression by sperm may allow for further MH discrimination leading to the cryptic female choice and selected abortion conserved amongst many species. Attempts to purify seminal MH I for 2D mass spectroscopy analysis, as well as antibody-based detection of MH II on the cell surface of trout spermatozoa are currently underway and will likely provide further insight into their function.

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Chapter 7

Out in the Cold:

Discussion, Conclusions and Future Aims

7.1. GENERAL DISCUSSION

7.1.1. Beta-$_2$-microglobulin

This light chain component of the MH I receptor is expressed in rainbow trout as a non-glycosylated 11kD polypeptide (Fig. 2-3), with highest relative expression in immune tissues (Fig. 2-2), as previously shown by northern blot analysis (Shum *et al,* 1996). Tissue distribution analysis also revealed β_2m is present in ovary lysates, as well as reproductive fluids. Due to its lack of a membrane-spanning domain, this protein is commonly found in both blood plasma and fluids, and is thought to be released during cell-surface MHC receptor turnover (Cooper *et al.*, 1984). Although it is critical to stable surface expression in mammals (Vitiello *et al.*, 1990), this MH I sub-unit is not expressed in carp subjected to 6°C for 6 days (Rodrigues *et al.*, 1998). Through the use of RT-PCR, relative β_2m transcript steady-state levels were found to be maintained in rainbow trout subjected to 2°C for 3 and 10 days (Fig. 2-1). Subsequent western blot analysis revealed that relative cellular protein steady-state levels were also maintained in all tissues and cells tested (Fig. 2-5). To assess surface expression, affinity-purified antibodies were employed against live peripheral blood leukocytes (PBL) isolated from fish after 10 days at 2°C and control temperatures (13°C). Indirect immunofluorescent detection using FITC-labeled anti-rabbit antibodies indicated comparable levels of β_2m on the cell surface at both temperatures (Fig. 2-6) Taken together, trout, unlike carp, appear to maintain MH I expression at low temperatures, likely due to adaptation to the selective pressures upon this cold-water species. Carp β_2m is a single copy gene (Dixon *et al.*, 1993); however twelve copies of the β_2m gene have been isolated from one individual trout (Magor *et al.*, 2004). Such an increase in gene copy number may provide a means for differential regulation and allow for one locus to compensate for the loss of another under a variety of conditions, such as low temperature. The promoter regions of both β_2m and MH I heavy chain have been cloned by our laboratory from rainbow trout, Atlantic salmon and the common carp. These promoters share numerous elements common to mammalian MH genes and are currently being incorporated into a self-constructed GFP-linked reporter construct. Future reporter assays may reveal the regions or factor(s) responsible for the differences that exist between trout and carp β_2m expression at low

temperatures. Such findings may also provide a means to explore the effects of immunostimulants or the development of transgenic fish stocks to enhance or convey temperature independent MH regulation.

7.1.2. MH I

Concurrent to the analysis of β_2m, antibodies developed towards a truncated form of trout MH I (UBA) revealed that MH I exists as an N-glycosylated polypeptide ranging in size from ~45 to 42kD throughout most tissues (Fig. 6-2). Like the light chain component, β_2m, transcript steady-state levels of MH I were found to be maintained in trout PBL at 3 & 10 days at 2°C (Fig. 7-1a). Similarly, subsequent western blot analysis of cellular protein steady-state levels (Fig. 7-1b) and indirect immunofluorescent analysis of cell-surface expression (Fig. 7-2) revealed that trout maintain MH I expression after 10 days at 2°C. In addition to the MH I protein shared among most tissues, the presence of a smaller, 35kD glycoprotein, that cross-reacted with the antiserum, was also detected in erythrocytes and some tissues (Fig. 6-2). Being too small to be the product of any known trout MH I allele, this polypeptide may represent the proteolytic digestion of a larger, full-length polypeptide or the product of an alternatively spliced mRNA, as previously seen in other species (Demaria & Bushkin, 2000; Roney *et al.,* 2004; Fujiki *et al.,* 2001). Western blot analysis also revealed the presence of MH I in both ovarian and seminal lysates, as similarly described in mammals (Martín-Villa *et al.,* 1999). What role these immune receptors may play in germ cell function remains unclear; however, the presence of a soluble MH I polypeptide in seminal fluid suggests a possible role in MH-based mate selection seen in fish and mammals (reviewed by Birkhead & Pizzari, 2002). Olfactory studies have demonstrated the ability of fish, including Atlantic salmon, to discriminate mate's MH genotypes (Landry *et al.*, 2001). Further studies are needed to assess this molecule's stability and potential role in mate selection, perhaps through mate preference to synthetic MH I allele types or antibody blocking experiments.

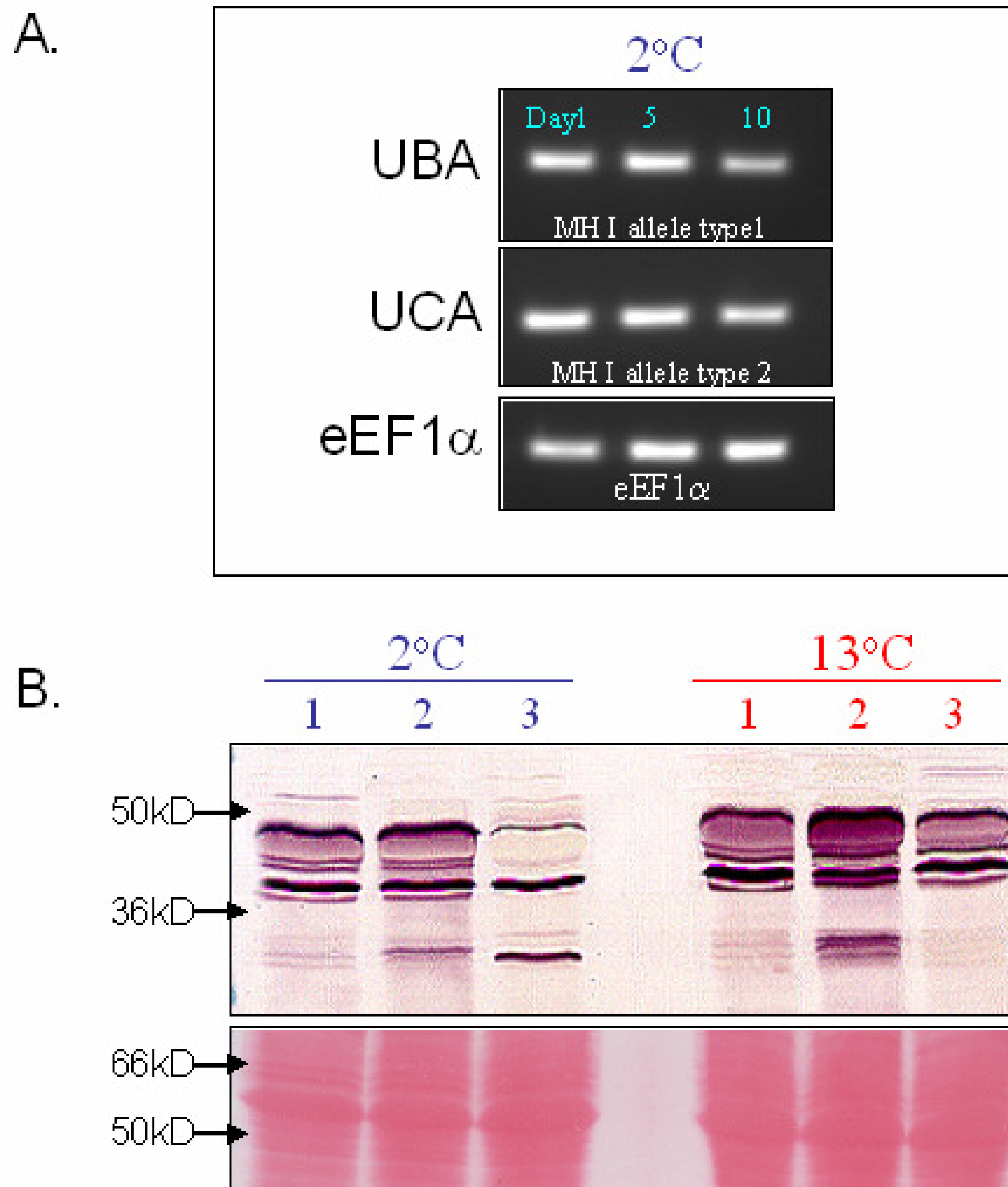

Figure 7-1. MH I transcript and protein expression at 2°C.

A. RT-PCR analysis of transcript steady-state levels in freshly isolated rainbow trout peripheral blood leukocytes (PBL) throughout 10 day challenge at 2°C, as described in chapter 2. Two oligonucleotide sets were designed against published sequence data to amplify known trout MH I heavy chain allele types, *Onmy*-UBA & *Onmy*-UCA, respectively. Elongation factor alpha served as an internal standard of RNA integrity and loading as previously described in Hansen & Strassburger, 2000. **B.** Western blot analysis comparing relative cellular MH I protein steady-state levels between 3 trout kept at 2° & 13°C for 10 days, as described in chapter 2. Ponceau S staining of membrane, shown below, indicates total protein transfer prior to membrane blocking and subsequent probing. Relative molecular weight standard sizes are indicated on the left.

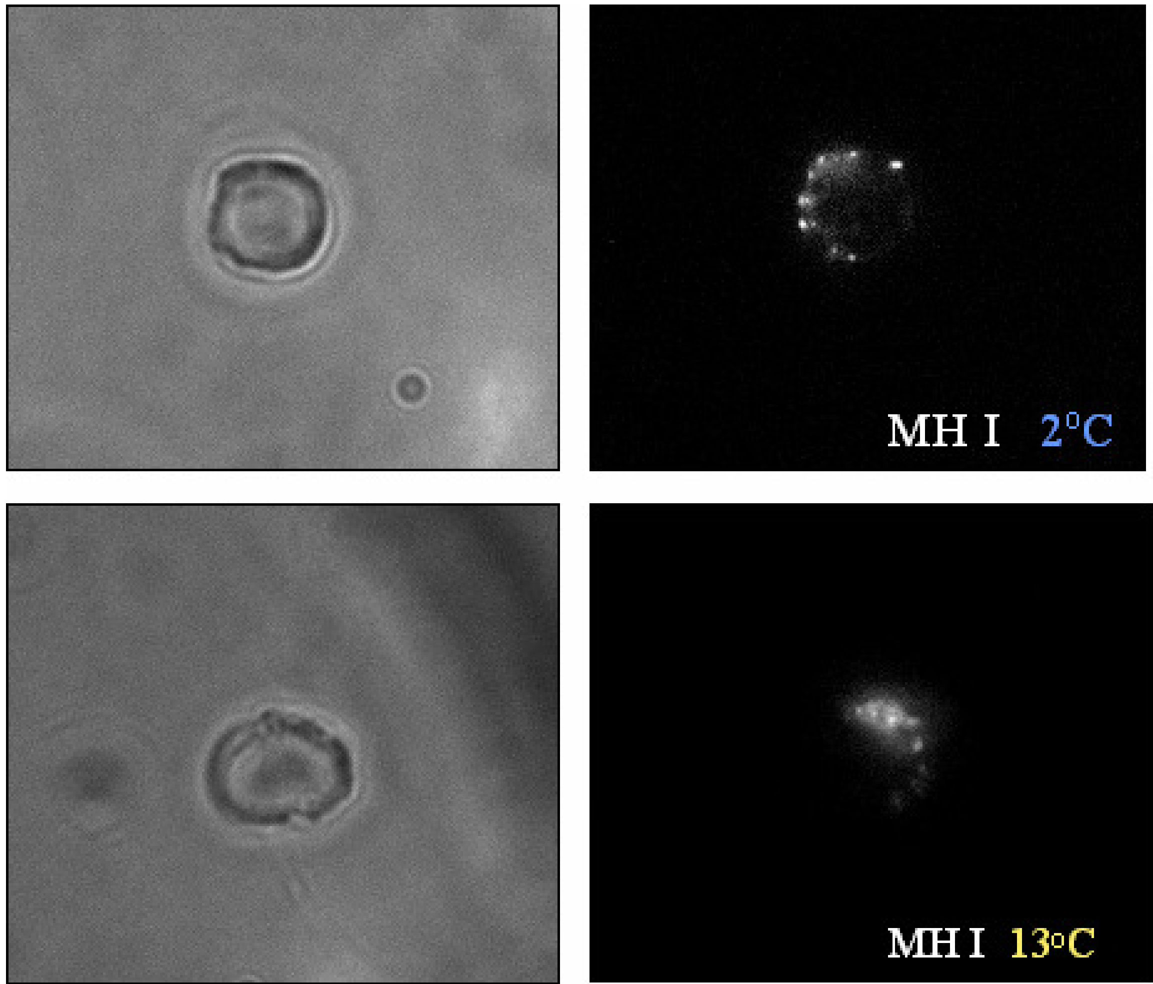

Figure 7-2. Surface MH I expression in trout PBL at 2° & 13°C.

Indirect epifluorescent detection of trout surface MH I after 10 days at 2 & 13°C. Peripheral blood leucocyte enhanced preparations were isolated from whole caudal blood of individuals kept at 2 & 13°C. Cells were then incubated with affinity-purified rabbit anti-trout MH I and detected using anti-rabbit IgG FITC, as previously described in chapter 2. Bound antibody, detected through an ultraviolet (u.v.) filter, served as a qualitative measure of surface expression. Panels on the left show bright-field images corresponding to u.v. images on the right. Negative controls included secondary antibody alone and 9E1, a monoclonal anti-catfish IgM with anti-mouse IgG FITC (not shown).

7.1.3. Calreticulin

The first calreticulin gene to be characterized in fish, the rainbow trout homologue appears to be a single copy gene based upon Southern blot analysis (Fig. 3-4a); however, phylogenetic analysis suggests that this may not be the case in zebrafish (Fig. 3-3). Trout CRT demonstrates 69% amino acid identity to both human and murine CRTs (Fig. 3-2). Like its mammalian counterparts, trout CRT protein is ubiquitous in its expression (Fig. 3-4b), with highest expression in liver and exists as a dimer with anomalous migration following SDS-PAGE (Fig. 4-1). Trout CRT did not appear to be glycosylated, though its sequence does contain a potential N-glycosylation site (Fig. 4-2). Despite the presence of ERSE sequences in its promoter (Fig. 4-3), trout CRT did not demonstrate detectable up-regulation by calcium homeostasis antagonists (CHA; Figs. 4-4 & 4-5). Though considered an ER resident protein, CRT has been identified on the surface of activated T cells in mammals (Arosa *et al.*, 1999). Through the development of polyclonal antibodies raised towards recombinant trout CRT, qualitative analysis of primary cultured trout PBLs, using indirect immunofluorescence, showed a similar increase in levels of cell surface expression following PHA activation (Fig. 4-6). Subsequent western blot analysis; however, revealed no detectable change in cellular protein steady-state levels (Fig. 4-6) suggesting that CRT may be regulated through cellular sub-localization in trout rather than by expression level, as seen in mammals (Waser *et al.*, 1997).

7.1.4. MH II[1]

Further supporting the conservation of MH receptor immune function in fish, genes encoding MH II α and β sub-units have been identified in several fish (reviewed by Shand & Dixon, 2001), as well as those encoding its accessory molecule, the invariant chain (Ii; Fujiki *et al.*, 2003). As the primary means of exogenous foreign peptide antigen recognition in adaptive immunity (Klein, 1986), both MH II α and β sub-units exist as ~35kD N-glycosylated polypeptides in trout immune tissues (Fig.7-3).

[1] Some of the work presented in this section has been published in *Immunogenetics* (2006) 58: 443-453 with co-authors S. Nath, K. Fujiki & B. Dixon

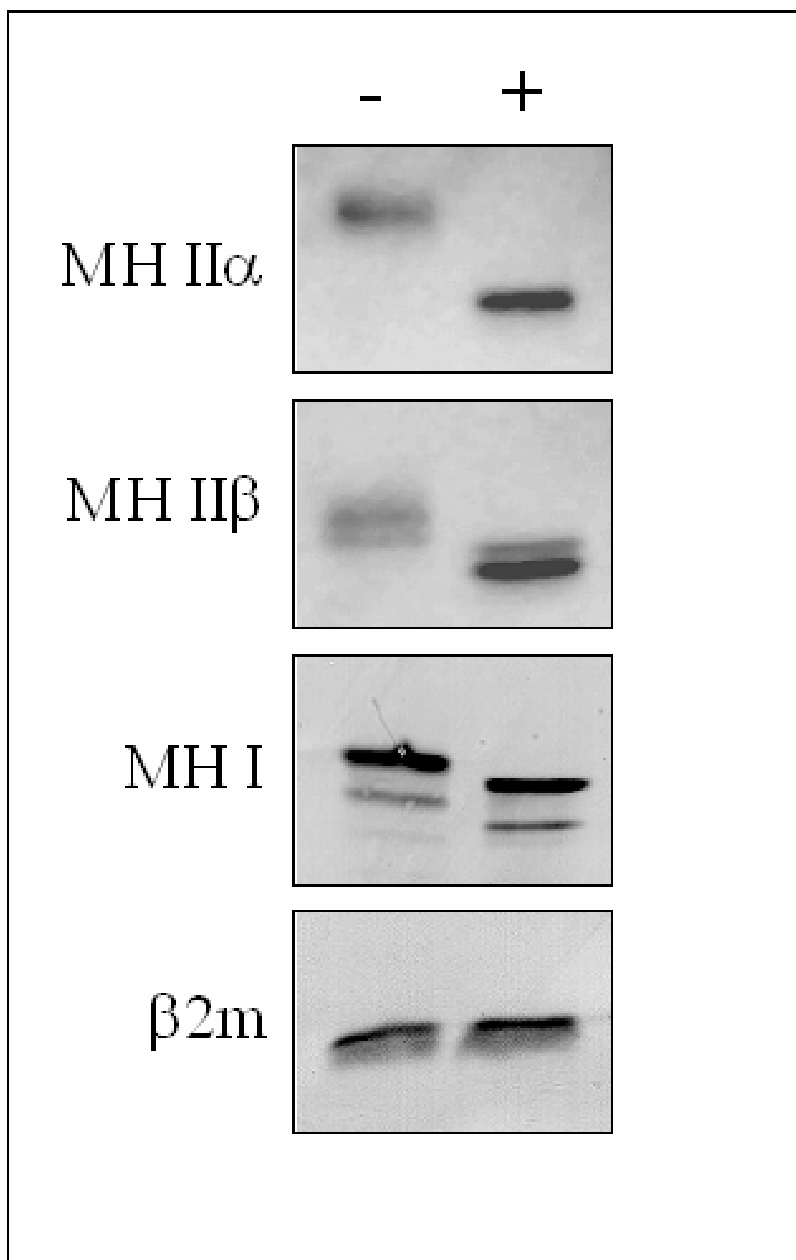

Figure 7-3. Western blot analysis of trout MH sub-units following *in vitro* de-glycosylation.

Aproximately 500µgs of rainbow trout gill lysate protein was boiled in denaturing buffer then incubated overnight with 2 500 U of Endo H (New England Biolabs). This was followed by a second overnight incubation with N-Glycosidase F (Roche). Mock reactions (-) contained equivalent volumes of water. Reaction volumes, equivalent to approximately 50µgs of original lysate protein, were separated by 15% SDS-PAGE and transferred to nitrocellulose for subsequent western blot analysis. Antisera used are indicated on the left. Mobility shift indicates de-glycosylation (+). β_2m, known not to any contain N-glycosylation sites, serves as a negative control.

Though the expression of these genes is maintained at 5°C, (not shown), both MH II α and β protein steady-state levels were dramatically down-regulated following exposure of trout to 2°C at 3 and 10 days, while β_2m was maintained, as previously reported (Fig. 7-4a). In addition, it should be noted that during early attempts at low temperature challenges, individual fish, weighing approximately 500g, were transferred to small, 10 gallon flow-through tanks. These tanks, being less than two fish lengths, greatly restricted fish movement during the challenge period. Subsequent western blotting revealed that both temperature-challenged and control fish demonstrated reduced MH II protein steady-state levels (Fig. 7-4b). Reduced MH II cellular protein levels continued throughout the 10 day challenge and subsequent 6 day recovery in both groups indicating that confinement, regardless of temperature, resulted in a loss of MH II cellular protein levels. Unfortunately, the antibodies developed against these recombinant trout MH II sub-units failed to react to native cell surface proteins using indirect immunofluorescence and therefore prevented their use in assessing MH II cell surface expression under low temperature and confinement. Despite this short-coming, the observed loss of cellular MH II sub-unit expression would prevent high levels of cell surface expression and therefore greatly hinder the presentation of subsequent foreign antigen and the ability to recognize extracellular pathogens, including bacteria or fungi. It is interesting to note that confinement stress, and the low temperature at which this down-regulation occurred, correlates with an increased frequency of several bacterial and fungal diseases (Woo & Bruno, 1999). *Flavobacterium psychrophillum*, the causative agent of cold-water disease and cold-water vibriosis demonstrates increased frequency at 3-5°C (Nematollahi *et al.*, 2003). The water mold, *Saprolegnia spp*. CF1 Bly has been isolated as the cause of "winter kill" in catfish farms following dramatic decreases in water temperatures (Bly *et al.*, 1992). These diseases afflict a wide range of fish and have accounted for significant economic losses in salmonid species. Such a dramatic loss of MH II expression in trout, as reported here, may provide one mechanism for this increased frequency of disease at low temperatures. Future efforts to clone these promoters may provide insight into their temperature-dependent regulation and allow for development of preventative strategies.

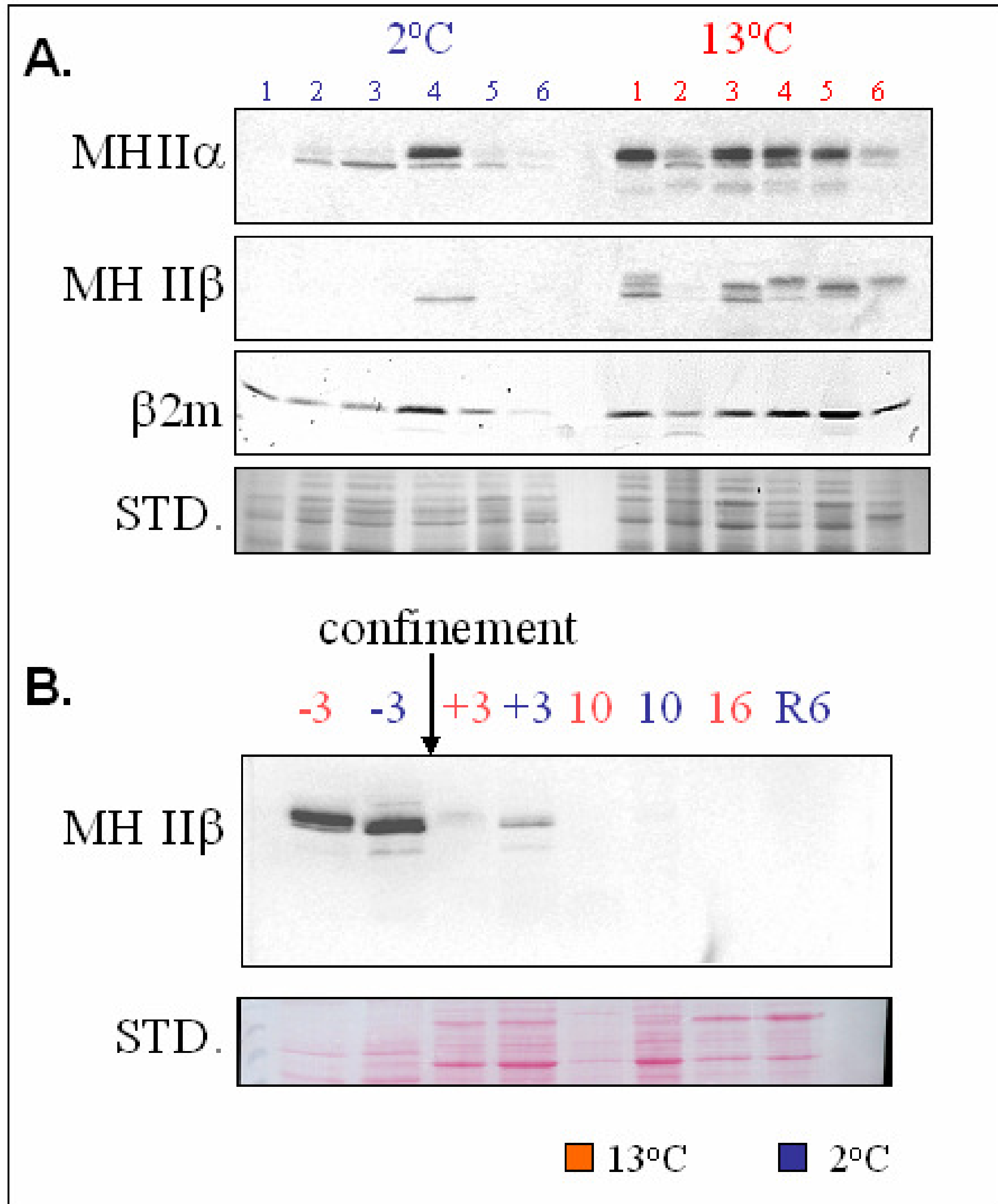

Figure 7-4. Trout MH II protein expression during low-temperature and confinement.

A. Western blot comparison of MH II α, β and β_2m cellular protein steady-state levels in PBL freshly isolated from 6 rainbow trout during challenge at 2°C versus 13°C controls. **B.** Western blot of MH II β protein steady-state levels in PBL of two individual rainbow trout during preliminary challenge experiments. Days with respect to tank transfer (stress) are indicated above, where -3 refers to PBL taken 3 days prior to transfer. Temperature challenge was initiated 24 hr. following transfer. Red text indicates control PBL isolated from one individual at control temperature, while blue text indicates PBL isolated from one individual at low temperature challenge. Following the 10 day challenge, tank water was returned to control temperature for 6 day recovery (R6). The control tank was maintained at ambient temperature throughout the 16 day experiment, as indicated (16).

7.1.5. Response of trout macrophage to a known fungal pathogen

Since its establishment in the late 1990's, the rainbow trout macrophage cell line, RTS11 has become a useful tool in studying trout immune function (Brubacher *et al.*, 2000; DeWitte-Orr *et al.*, 2005). As the cause of "winter kill" and numerous fish mycoses worldwide (Bly *et al.*, 1992) as well as their ease of use under laboratory conditions, the saprolegniales seemed a natural choice for use in studying trout macrophage response *in vitro*. *Achlya bisexualis* and *Saprolegnia parasitica* are two members of the Saprolegniales and have been shown to infect fish (Jeney & Jeney, 1995). Both live and heat-killed cultures of *A. bisexualis* and *S. parasitica* induced homotypic aggregation (HA) in RTS11 within 48 hr. of mixed culture (Fig. 5-1). HA appeared to be enhanced when using conditioned media from co-cultured RTS11 and *Achlya*, suggesting the presence of synergistic effectors of aggregation (Fig. 5-5). The presence of fungal toxins was not evident and phagocytosis, the primary function of macrophage, appeared inhibited by the large fungal spore diameter. Typically restricted to tumour and viral peptide recognition (Klein, 1986), MH I and its accessory gene transcripts appeared unchanged in RTS11 when in the presence of the mold; however the pro-inflammatory genes: Cox-2, TNFα and IL-1β were strongly up-regulated (Fig. 5-6). Most notable was the dramatic down-regulation of MH II α and β transcripts, as well as those of its accessory molecule, the invariant chain (Fig. 5-6). As the primary means of exogenous pathogen recognition, such a dramatic loss of MH II expression *in vivo* would impair the ability to present fungal antigens to lymphocytes, thereby providing a means to evade one arm of this host's defenses. A similar study using *Aphanomyces*, showed that salmon head kidney macrophage could eliminate low spore concentrations through phagocytosis (Miles *et al.,* 2001); however the larger spore diameter, seen in the saprolegniales, greatly impairs the ability for a similar spore clearance prior to their germination.

7.2. CONCLUSIONS

The data presented in chapters 3 & 4 indicates that trout calreticulin, though similar in many aspects to its mammalian counterparts, is not strongly up-regulated upon various CHA treatments. What role this highly conserved protein plays in fish is still unclear; however, data suggests that its expression or functions may be regulated by a different mechanism. Stress response elements, originally identified in mammals are conserved in trout, suggesting it does play a role in the unfolded protein response; however this has yet to be demonstrated. Co-immunoprecipitation and sub-cellular localization studies will undoubtedly provide more insight into the role of this highly conserved, yet equally mysterious protein.

Based upon the work presented in chapters 2 & 7, MH I and β_2m expression levels are maintained in trout kept at low temperatures. These results differ from the temperature-dependent regulation observed in carp and suggest that components responsible for viral recognition are in place at low temperatures. Whether these receptors maintain the capacity to present endogenous (viral) antigen to circulating T lymphocytes at this low temperature, has yet to be determined. Future *in vitro* pulse-chase studies employing labeled antigenic peptides and/or supplemented growth medium may provide valuable information regarding the cell-surface turnover and functionality of these receptors at low temperature.

As demonstrated in chapter 5, MH II transcript levels in the trout macrophage cell line, RTS11 were dramatically reduced when cultured in the presence of a common fungal pathogen. Interestingly, this pathogen is known to afflict fish following severe reductions in water temperature (Bly *et al.,* 1992). As the primary means of fungal and bacterial recognition, the dramatic down-regulation of MH II shown above (Fig. 7-4) following low temperature challenge (2°C) or confinement, clearly provides a means for these pathogens to evade host defenses during the establishment of infection. This loss of expression, due to low temperature, was observed only *in vivo* (Nath *et al.,* 2006) indicating that factors outside of cell culture conditions likely play a role in this temperature-dependent regulation.

During outbreaks of "winter kill", Bly and others reported that mass mortality occurred during a return to warmer temperatures. Though numerous factors can contribute to the rate and severity of infection, based upon the data reported here, such a dramatic reduction in water temperature may result in the down-regulation of MH II expression in trout leukocytes, as reported here, and allow for the establishment of *Saprolegnia* infection. A return to warmer water temperatures would provide the return of basal MH II expression, but also increased fungal growth. The growing fungus, resistant to macrophage phagocytosis, as demonstrated in chapter 5, would induce a pro-inflammatory response, but also lead to a continued down-regulation of MH II and thereby providing a prolonged evasion of antigen recognition and antibody production, which is likely required for large spore phagocytosis (Canon & Swanson, 1992). With little opposition, this mold would eventually invade visceral organs, as previously described (Neish, 1977) leading to mortality.

Taken together, the work presented here demonstrates that these cold-water species maintain at least some of the key components required for viral and tumour recognition at low temperatures; however during times of stress and low temperatures, these fish may become increasingly susceptible to extracellular pathogens, such as bacteria and fungi. Once established, these pathogens have the ability to suppress this sensitive component of adaptive immunity. Such knowledge may allow for further development of disease prevention and treatment protocols. Future cloning and subsequent study of trout MH II promoter elements may identify transcription factor(s) which may be responsible for reduced expression under low temperature and confinement. One factor of immediate interest is the class II transactivator, CIITA. In humans, this factor utilizes 4 separate promoters for the independent expression of MH I & II. If conserved in trout, reduced expression of this critical factor may originate in one of these promoter regions. These studies and the effects of immunostimulants may facilitate enhanced breeding programs and disease treatments.

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