

**Impact of Temperature on the Antigen Presentation pathway
in VHSV-infected Rainbow Trout (*Oncorhynchus mykiss*)**

by

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A thesis

presented to the University of Waterloo

in fulfilment of the

thesis requirement for the degree of

Doctor of Philosophy

in

Biology

Waterloo, Ontario, Canada, 2025

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This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of Contributions

Chapter 3 – RTGut-GC cell line results were generated in collaboration of R. Supinski as part of their BIOL 499 undergraduate thesis. These data were incorporated into this thesis with appropriate permissions, and additional analyses were conducted.

T. Rodríguez-Ramos developed and optimized the sandwich ELISA for the IFN-I analysis.

Chapter 4 – The initial optimization of fin cell cultures and standardization of the antigen presentation assay was in collaboration with F. Barraza-Rojas and M. Imarai, using other fish pathogens.

T. Rodríguez-Ramos developed and optimized the sandwich ELISA for the IFN γ and IL-2 analysis.

Abstract

Sudden water temperature shifts are increasing in frequency and altering aquatic ecosystems, where small water bodies are especially vulnerable. These rapid temperature drops can severely impact fish immune system, and can also influence host-pathogen interactions, particularly during viral infections. Rainbow trout (*Oncorhynchus mykiss*) is economically important in aquaculture and is highly susceptible to disease outbreaks such as viral hemorrhagic septicemia virus (VHSV). A key process during antiviral immunity is the antigen presentation by the major histocompatibility complex class I receptor (MHC-I) and its chaperone β 2-microglobulin (β 2m). These proteins form a trimeric complex with viral peptides that can be presented to and recognized by CD8⁺ T cytotoxic cells, which activate adaptive immunity. While transcriptional responses of antigen presentation molecule genes to low temperatures combined with VHSV infection have been previously demonstrated, little is known about the folding and stability of the MHC-I complexes, the antigen processing and presentation processes, and T cell activation of an infection with VHSV during thermal stress. This thesis investigates how suboptimal temperatures modulate the antigen presentation pathway in rainbow trout infected with VHSV, integrating bioinformatic analyses, *in vitro* refolding of the MHC-I complexes, *in vitro* cellular responses and T cell activation using a novel antigen presentation assay.

Initially, the thermostability of the rainbow trout MHC-I UBA molecule complexed with β 2m and VHSV-derived peptides was evaluated. Peptide candidates derived from VHSV nucleoprotein (Protein N), and glycoprotein (Protein G) were selected using a combination of bioinformatic tools and then, the predicted structure of the MHC-I/ β 2m/VHSV-peptide complex

was analyzed using AlphaFold2. Temperature influenced the structural behavior of the MHC-I complexes according to a computational simulation program revealing potential molecular mechanisms of thermal stress on the antigen presentation pathway. To validate these *in silico* analysis, MHC-I and β 2m recombinant proteins were successfully purified using *E. coli* expression systems, and *in vitro* refolding with the selected VHSV peptides was optimized. These results provided experimental evidence of rainbow trout MHC-I complexes refolding with VHSV peptides for the first time. Differential scanning fluorimetry confirmed that the stability of these complexes was sensitive to temperature and dependent on both peptide binding and biochemical characteristics.

Continuing with the molecular characterization of this pathway, the functional effects of temperature on antigen processing, trafficking and presentation was assessed using a controlled *in vitro* system with VHSV-infected rainbow trout cell lines; RTGill-W1, RTS11, and RTGut-GC. These cells were infected and exposed to suboptimal temperatures, 4°C and 14°C, for nine days and, 20°C-exposed cells were used as control group . Flow cytometry analysis showed that the cell surface expression of MHC-I/ β 2m was reduced during infection at 14°C, compared to other temperatures. In addition, these results correlated with the release of β 2m to the extracellular space. On the contrary, proteasomal activity and the secretion of the antiviral cytokine IFN-I were not impaired at suboptimal temperatures, highlighting the specificity of temperature-sensitive antigen presentation disruption.

Finally, an antigen presentation assay using dorsal fin cells as antigen presenting cells was developed and validated to analyze the impact of temperature in antigen recognition and T cell activation. To perform this, primary fin cell cultures were exposed to heat-killed VHSV and co-

cultured with VHSV-sensitized splenocytes. Cytotoxicity was evaluated with the release of lactate dehydrogenase (LDH), resulting in an antigen-specific and MHC-I-dependent cytotoxic response from the activated splenocytes. In addition, this cytotoxic response was negatively affected by lower temperatures, once again, supporting the idea that suboptimal temperatures impair the antigen presentation pathways at different levels during a viral infection in rainbow trout.

Altogether, this thesis offers a comprehensive and integrated approach into understanding how temperature regulates antigen presentation during viral infections in rainbow trout. It highlights not only the thermal sensitivity of antigen presentation and viral antigen processing but also contributes to the immense knowledge gap about fish immunity. Importantly, these findings provide *in silico* and *in vitro* methodologies for studying antigen presentation and T cell cytotoxicity, with direct application for vaccine design and maintaining fish health, especially during thermal stress.

Acknowledgments

First, I would like to express my deepest gratitude to my supervisor, Dr. Brian Dixon. Your guidance and encouragement throughout this journey have been invaluable. You have not only guided me in the lab and through my research but also supported me through many challenges outside academia. You always believed in my work, gave me the freedom to explore, and always pushed me to keep going.

I would also like to thank my committee members, Dr. Marc Aucoin and Dr. Todd Holyoak, for their time, insightful feedback and thoughtful questions throughout the development of this work. You challenged me to think critically and pushed me to improve my work. I am grateful for the opportunity to have learned from each of you. Also, to Dr. Holyoak's lab members for helping me with some challenging experiments.

To my lab mates and colleagues in the Dixon lab, thank you for the shared experiments, coffee breaks and laughs that made the days more bearable. A very special thanks to Tania; even though I forced you to go to the gym, you were always (or most of the time) kind to me, and your friendship made a lasting difference during the most intense periods of this PhD. Your support both in and out of the lab kept me going so I'm endlessly grateful. Also, to Rochelle for making the lab a fun place to work in.

To my brothers, sister-in-law, and friends, and specially to my mom and dad – thank you for being my foundation. Your love, patience and belief in me gave me the strength to keep going through the challenges and self-doubt. Thank you for always supporting me and reminding me of who I am outside of the lab and helping me find balance when I needed it the most, especially

when I wanted nothing more than to quit and go back home to eat empanadas, sopaipillas and cazuela.

Obviously, thanks to my spouse who came with me to Canada without hesitation and made sacrifice after sacrifice so I could finish my degree. You taught yourself to learn a new language and even learned to love the Canadian cold winters, which now that I think of, is very convenient for you since is a perfect time of the year for you to play videogames all day. I can't put into words how much it means to me every late night and mental breakdown that you were next to me giving me the strength to keep going. I'd like to say that this degree will solve the fact that we're poor, but you and I both know that is not true; however, I can promise that I will keep loving you more and more every day.

To Pupo – what can I say? We've known each other for many years and somehow decided, because we hate ourselves, to take on this PhD madness together in different hemispheres. Having someone with the same mental deficiencies and who understands exactly how draining this is has made all this journey bearable. Talking and complaining to you every day about our lovely lab experiences has meant everything to me. I know that everything seems very dark right now, but I promise you that this will be behind us very soon and we will have the chance to meet up again and be happy, or maybe I am just saying this because I am confused, but anyways te estimo amiko.

And finally, to the amazing people I met here in Canada, specially to my adopted children Tati and Kevin, my friend Vari and the duo Gordo and Rafa. They dragged me out of the lab to have fun and remember that life is more than just working. You helped me stay sane and reminded me to laugh when everything felt heavy.

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List of Abbreviations

APC: antigen presenting cell

β2m: β2-microglobulin

CD8: cluster of differentiation 8

ECL: enhanced chemiluminescence

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

EPC: epithelioma papulosum cyprinid cell line

ER: endoplasmic reticulum

FBS: fetal bovine serum

GdnHCl: guanidine hydrochloride

IFN-I: type I interferon

IgG: immunoglobulin G

IgM: immunoglobulin M

IgY: immunoglobulin Y

IL-2: interleukin 2

ISG: interferon-stimulated gene

L-15: Leibovitz's L-15 medium

LB: Luria-Bertani medium

MHC-I: major histocompatibility complex class I

MOI: multiplicity of infection

Ni-NTA: nickel-nitrilotriacetic acid

NaCl: sodium chloride

OD: optical density

PBS: phosphate buffered saline

Pfu: plaque forming units

PRR: pattern recognition receptors

RIG-I: Retinoic acid-inducible gene I

RTGill-W1: rainbow trout gill cell line

RTGut-GC: rainbow trout gut cell line

RTS11: rainbow trout spleen cell line

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOC: Super Optimal broth with Catabolite repression medium

TAP: transporter associated with antigen processing

TBS-T: Tris-buffered saline with Tween 20

TCA: Trichloroacetic Acid

TCEP: Tris(2-carboxyethyl)phosphine

TCID₅₀: median tissue culture infectious dose

TCR: T cell receptor

TLR: toll-like receptor

TMB: 3,3',5,5'-Tetramethylbenzidine

TBS-T: Tris-buffered saline with Tween 20

VHSV: viral hemorrhagic septicemia virus

Chapter 1: General introduction and thesis scope

1.1 Introduction

1.2 Aquaculture industry and the disease problem

Aquaculture, the farming of aquatic organisms, is one of the fastest-growing industries in global food production. As the global population increases, aquaculture is now seen as a key solution to meet the growing demand for seafood (FAO, 2024; Little et al., 2016). Rainbow trout (*Oncorhynchus mykiss*) is a species that has rapid growth, adaptability and brings a high price so it plays an important role in the global aquaculture industry, especially in North America, Europe, and parts of Asia and South America (MacCrimmon. R, 1971). This species is widely distributed in Canada, where it can be found in various streams, rivers, lakes and ponds along the Pacific coast, and it has also been introduced in the Great lakes area (Fisheries and Oceans Canada (DFO), 2016; Government of Canada, 2023). A common method of farming rainbow trout in these regions involves using net-pen systems suspended in water bodies such as lakes and reservoirs. These systems are preferred to cultivate species such as rainbow trout because it provides continuous water exchange with the natural environment that is suitable for farming large groups of fish (Borland, 2023).

Despite the projected growth of the aquaculture industry, it faces many challenges, specifically due to disease management (Huntington et al., 2021). Diseases provoked by viruses, bacteria or parasites cause elevated economic losses of \$6 billion USD per year, with a projection of \$10 billion dollars by 2030 (Calvin et al., 2023). These outbreaks not only impact fish health but also disrupt production cycles (Cain, 2022; Mugimba et al., 2021). The relationship between environmental factors and disease outbreaks is becoming more evident, with temperature

recognized as a key modulator of fish physiology, immune function, and disease susceptibility (Figure 1.1) (Bailey & Secor, 2016; Reid et al., 2022). As ectothermic animals, fish such as rainbow trout depend on external environmental temperatures to regulate their body temperature. This makes them more sensitive to temperature fluctuations, which can directly affect their physiological processes and ability to fight infections (Haesemeyer, 2020; Rodgers, 2021). For rainbow trout, the optimal temperature range for normal physiological and metabolic function is around 12-18°C (Jiang et al., 2021). During heatwaves, elevated water temperatures can stress fish, impair their immune system, and make them more susceptible to infections (Haesemeyer, 2020; Mariana et al., 2019; C. Yang et al., 2022). Similarly, sudden drops in temperature during cold winters can suppress the immune system of fish and potentially trigger disease susceptibility and impairing their ability to recover from infections (Michie et al., 2020; Vadboncoeur, Nelson, Clow, et al., 2023; Vadboncoeur, Nelson, Hall, et al., 2023). In both natural and aquaculture systems, water temperatures can fluctuate rapidly, changing by several degrees in hours due to factors such as weather changes, and water flow dynamics. These fluctuations can be more drastic in net-pen systems where fish are exposed to surface water temperatures, increasing their susceptibility to disease outbreaks (Moccia et al., 2010; Trumpickas et al., 2015).

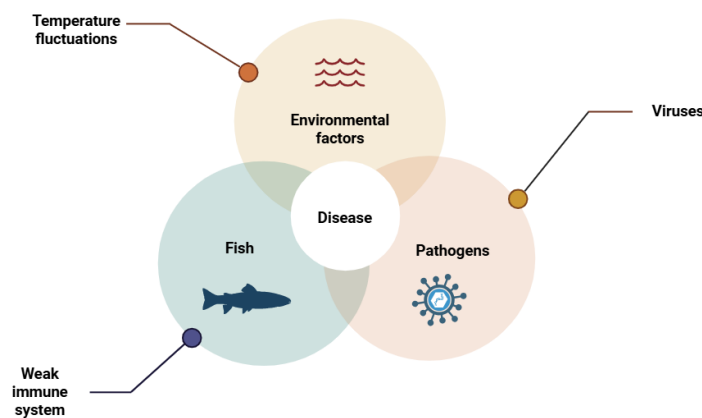


Figure 1.1. Disease outbreaks. Factors determining disease outbreaks in aquaculture (adapted from Moreira et al., 2021). Created in BioRender.com.

Vaccination is widely used to protect farmed fish against pathogens, including viruses, however, the efficacy of some vaccines is inconsistent and can be affected by several factors, with water temperature being the most critical (Adams, 2019; Kumar et al., 2024; Mkulo et al., 2024). Optimal immune responses to vaccination happens in a specific temperature range that increases antigen uptake, processing and the activation of both innate and adaptive immune molecules. Therefore, using vaccines during periods with temperature fluctuations or outside the optimal temperature range for each species can affect negatively the vaccine efficacy. As a result, is necessary to consider the seasonal temperature fluctuations and the effects of climate change when planning on vaccination schedules (Köllner & Kotterba, 2002; Lorenzen et al., 2009; Martins et al., 2011; Raida & Buchmann, 2008; Semple et al., 2025; J. Wang et al., 2020). In addition, understanding how pathogens behave in different temperature conditions is important, because temperature can affect their virulence, replication rates and transmission dynamics (Combe et al., 2023; Lusiastuti et al., 2024; Okon et al., 2024). Due to these challenges, a deeper understanding of the fish immune system and host-pathogen interactions is essential. Elucidating how immune responses are regulated under thermal stress and how pathogens exploit weakened defenses is critical for the development of effective tools to minimize disease outbreaks and promote sustainable aquaculture practices.

1.3 Viral hemorrhagic septicemia virus

The viral hemorrhagic septicemia virus (VHSV) is one of the deadliest infectious diseases that affects a variety of fish species including rainbow trout, generating high economic losses in fish farms (Skall et al., 2005). Among the various strains of VHSV (genotypes I, II, III, IV) (Einer-Jensen et al., 2004; Elsayed et al., 2006), genotype IV has been found in Canada, specifically in the Pacific and Atlantic coasts as well in the Laurentian Great Lakes. Within genotype IV, subtype IVa has been identified in both the east and west coasts (Garver et al., 2013; Meyers & Winton, 1995), while IVb is widely found in the Great Lakes region (Gagne et al., 2007; Thompson et al., 2011; Winton et al., 2008), and IVc is most restricted to New Brunswick and Nova Scotia (Pierce & Stepien, 2012a). The last large outbreak of genotype IVb was recorded in 2000 where several mortalities were observed in freshwater fish species in the Great Lakes region of North America (R. Kim & Faisal, 2011). Additionally, a recent and unusually high number of fish mortalities in Lake Huron has been associated with a potential outbreak caused by this virus during the early spring of 2025 (Butler, 2025). The transmission of this virus is primarily horizontal because of the excretion of virus from infected fish which contaminate the water, leading to the entrance of the virus into the host through the gills, wounds on the skin and also fin bases (Harmache et al., 2006). VHSV belongs to the Rhabdoviridae family, and the genome is formed by a non-segmented negative-sense single stranded-RNA molecule of ~11 kb encoding six viral proteins. The viral RNA is encapsulated with a nucleoprotein (N), a polymerase-associated phosphoprotein (P) and the large RNA-dependent RNA polymerase (L) that forms a ribonucleoprotein complex (RNP), which interacts with the matrix protein (M). The entrance of the virus to the host cells is due to the viral surface glycoprotein (G). The last protein is encoded by a gene localized between the G and

L genes, resulting in the expression of a non-structural NV protein (non-virion) (Schutze et al., 1999). As previously mentioned, temperature not only influences the ability of fish to fight infections but also plays a critical role in pathogen behavior. For viral hemorrhagic septicemia virus, the optimal temperature range for initiating infection is between 9-15°C. The virus's survival outside the host is also temperature-dependent, with 4°C giving optimal conditions compared to warmer temperatures where its stability declines. High infectivity rates and viral replication within the host have been observed between 13-20°C, while transmission is more effective at temperatures ranging from 1-12°C (S. J. Kim et al., 2019; S. J. Kim & Oh, 2020). Therefore, understanding the temperature-dependent behavior of pathogens such as VHSV is essential for predicting disease risk and implementing effective health management strategies in aquaculture.

1.4 Antigen processing and presentation

1.4.1 Antigen presentation pathways in mammals

The immune system relies on a process called the antigen presentation pathway to detect viruses and prevent their propagation in the host (Croft et al., 2019). The main molecule involved during this process is the major histocompatibility complex class I (MHC-I). The MHC-I molecule play an indispensable role in the adaptive immune response by inducing cell-mediated immunity to eliminate pathogen infected cells or cells producing mutant proteins (Pishesha et al., 2022). MHC-I molecules are expressed in all nucleated cells and in the endogenous antigen presentation pathway, they present cytoplasmic antigens to CD8⁺ T cells also known as cytotoxic T lymphocytes (CTL). This interaction between the antigen presenting receptor, MHC-I, and the T Cell receptor, plus the co-receptor CD8, on cytotoxic T lymphocytes triggers the activation of the

CTLs which generates the eradication of infected cells in all tissues (Pishesha et al., 2022; Salter Russel D et al., 1989). The processing and presentation of antigens to CD8⁺ T cells triggers key elements of adaptive immunity such as specificity and memory to specific antigens. However, the antigen repertoire presented by MHC-I molecules depends on the products formed by cytosolic proteolysis, which can come from misfolded proteins, or intracellular antigens from pathogens like viruses (Wearsch & Cresswell, 2013). These antigenic peptides are generated through the ubiquitin-proteasome system and the resulting peptides are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) (Santambrogio et al., 2019). The proteasome-derived peptides can be trimmed by the endoplasmic reticulum aminopeptidase 1 (ERAP1). The specific aminopeptidase activity of ERAP leads to precursors of eight to ten amino acid residues, which is the optimal length for binding to MHC-I molecules. Loading of these trimmed peptides leads to the presentation of the MHC-I molecule/peptide complex on the cell surface (Mpakali et al., 2019).

The proper folding, stabilization and assembly of the MHC-I molecules is crucial for the loading of high-affinity trimmed peptides. This process is coordinated by several ER chaperones, which form the multi-protein complex called the peptide-loading complex (Pishesha et al., 2022). Initially, newly synthesized MHC-I heavy chain associated with the ER chaperone β 2-microglobulin (β 2m), then this complex is stabilized by additional ER chaperones. Peptide loading is then facilitated by tapasin, a critical component of the PLC that promotes the selection and binding of high-affinity peptides (Fisette et al., 2016). Once fully assembled, the heterotrimeric complex peptide-MHC/ β 2m is transported through the Golgi apparatus to the cell surface for antigen presentation to CD8⁺ T cells (Figure 1.2) (Blees et al., 2017a; Panter et al., 2012). After

antigen presentation and recognition by CD8⁺ T cells, several molecules are expressed and release to activate the immune system to response and help to prevent viral propagation. Among these molecules are cytotoxic killing effectors and cytokines which triggers programmed cell death of infected cells, stopping viral infections (Sigal, 2016).

Like many cell surface receptors, MHC-I molecules are recycled within the cell, during and/or after antigen presentation, into endosomal or degradation compartments. During this internalization process, MHC-I complexes can acquire new antigenic peptides with higher affinity, and the be transported to the cell surface to re-initiate antigen presentation. On the other hand, sub-optimally loaded MHC-I, which are considered unstable, are transported to lysosomes for further degradation. When these sub-optimally loaded MHC-I complexes are presented on the cell surface, part of the bound β 2m is released into the extracellular space as MHC-I turnover marker and quality control system of the antigen presentation pathway (Figure 1.2) (Grommé et al., 1999; Montealegre et al., 2015; Montealegre & Van Endert, 2019b).

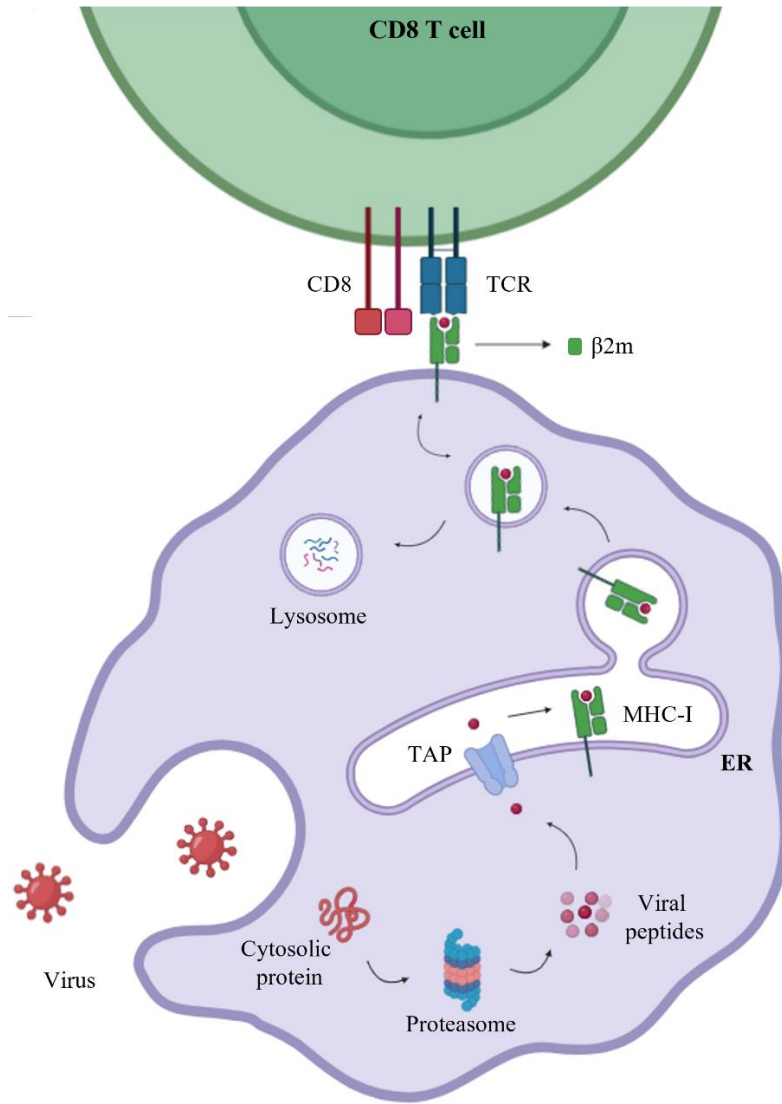


Figure 1.2. Antigen presentation pathway. During a viral infection, processed viral peptides by the ubiquitin-proteasome system are loaded onto MHC-I molecules and then transported to the cell surface. On the cell surface, viral peptides presented by MHC-I molecules are recognized by CD8⁺ T cells triggering an adaptive immune response against the viral infection. The MHC-I complexes are recycled through the cells, releasing the ER chaperone β2m into extracellular space. *Created with BioRender.com.*

1.4.2 Antigen presentation pathway in Rainbow trout

In humans, the major histocompatibility complex region is on the short arm of chromosome 6 and is one of the most gene-dense regions of the genome (~ 4 Mb). This multigene complex encodes class I molecules as well as other components of the innate and adaptive immune responses (Cunliffe & Trowsdale, 1987). Differently from humans, MHC class I genes in Rainbow trout (*Oncorhynchus mykiss*) are not linked together in the same chromosome, leading to the conclusion that in bony fish the major histocompatibility genes do not form a complex (Dixon et al., 1995a; Phillips et al., 2003; Shum et al., 1999). In rainbow trout there are two separate regions encoding MH class I molecules called, *Onmy-IA* and *Onmy-IB*. The latter is where the classical class I gene *UBA* is expressed in the *Onmy-IA* locus while four non-classical genes, *UCA*, *UDA*, *UEA* and *UFA*, are encoded in the *Onmy-IB* region (Shiina et al., 2005). The most common allomorph corresponding to the classical *Onmy-IA* is the molecule *Onmy-UBA*501*, which has been found expressed in different rainbow trout wildtype strains and, also in several tissues and cells (Dijkstra et al., 2003a; Fischer et al., 2005).

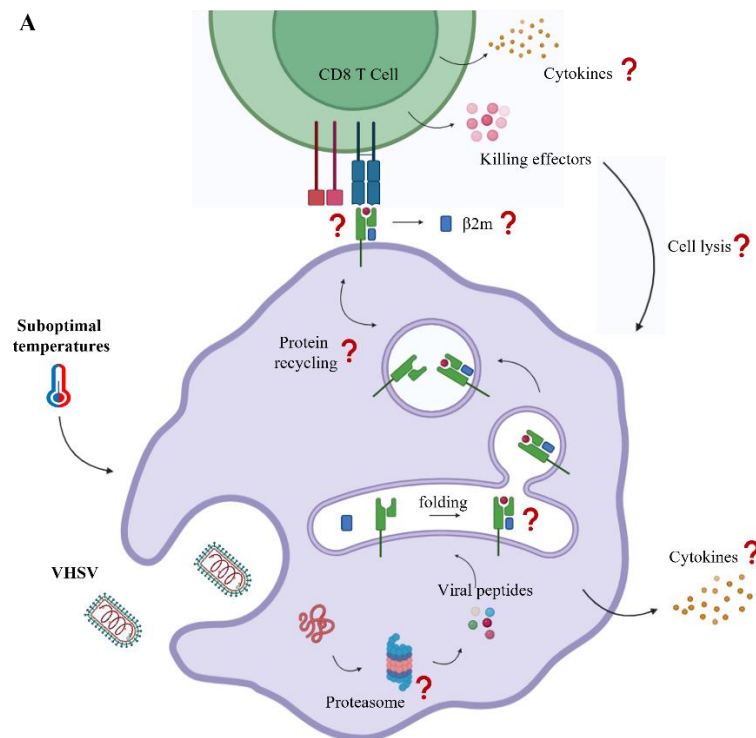
Molecular cloning and gene expression analysis of the MH class I molecule and the peptide loading complex have been performed in rainbow trout. The gene pattern of $\beta 2m$ has been studied and three polymorphic genes, which differ in regions that do not interact with the MH class I heavy chain, have been described (Magor et al., 2004). The amino acid sequence of $\beta 2m$ has a 69% identity with murine protein (Choi et al., 2006) and its protein expression have been observed in several tissues such as gill, spleen, intestine, kidney, muscle, and liver (Kales et al., 2006). The peptide-proof editor, tapasin, have been identified to be encoded by two genes in rainbow trout and its gene expression was demonstrated to be induced during the infection. The amino acid

sequence of tapasin shares an identity of 29% with human and murine protein (Landis et al., 2006). Tapasin protein expression has been found in thymus, kidney, spleen, gill and also in several rainbow trout cell lines (Sever et al., 2018a).

Several studies have shown that the antigen presentation pathway in fish is impaired under suboptimal temperatures (Rodrigues et al., 1998; Lumsden, et al., 2014; Abram et al., 2019). It has been observed that during VHSV infection, the intracellular protein levels of the MH class I heavy chain, $\beta 2m$ and the chaperone tapasin, are increased 14 d after infection in a rainbow trout spleen cell line (RTS11) at 14°C, however, no differences were observed when cells were exposed to 2°C (Sever, Vo, Lumsden, et al., 2014). In a similar trend, rainbow trout skin cell line, RTDHF, had a delayed up-regulation of *mhcI* expression at 4°C compared to 20°C (Abram et al., 2019).

Despite these advances, there is still a knowledge gap in how suboptimal temperatures affects the folding of the MHC-I/ $\beta 2m$ /peptide complexes and, also, how it affects the intracellular dynamics of antigen processing and antigen presentation. In addition to this, it is not known how temperature fluctuations affect antigen recognition and T cell activation in rainbow trout infected with VHSV. For this reason, this thesis is organized into three chapters, all centered on how suboptimal temperatures impact the antigen presentation pathway in rainbow trout during a viral infection with VHSV (Figure 1.3). It is important to mention that suboptimal temperatures refers to any temperature condition that deviates from the optimal temperature for the rainbow trout cell lines and/or primary cell cultures used in this thesis. Each cell tested in this thesis has different optimal temperature for normal growth and cellular metabolism and lower/higher temperatures from this optimal were used to induce thermal stress in each chapter.

Regarding the thesis outline, chapter two focuses on how suboptimal temperatures affects the folding of MHC-I molecules complexed with VHSV peptides. Chapter three investigates the processing of viral antigens, and chapter four, T cell activation after viral recognition. This study will provide valuable knowledge about the antigen presentation pathway in rainbow exposed to suboptimal temperatures during an infection with the viral hemorrhagic septicemia virus (VHSV). In addition, this study will offer important insight into temperatures ranges required for effective antigen presentation and T cell activation, which could serve as guidance for determining optimal temperatures at which vaccines elicit immune responses in fish.



B

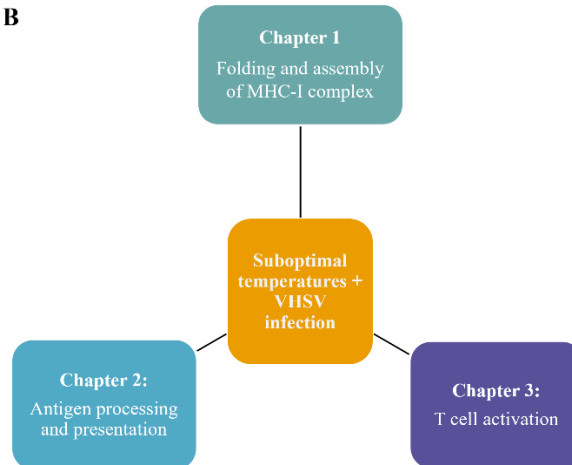


Figure 1.3. Thesis outline diagram. The present thesis will address how suboptimal temperatures affects different steps of the antigen presentation pathway in rainbow trout during an infection with VHSV (A). The thesis was separated into three chapters analyzing from folding of MHC-I complexes, followed by antigen processing and ending in T cell activation (B). *Created with BioRender.com.*

1.5 Thesis Hypotheses

Suboptimal temperatures impair MHC-I antigen presentation in rainbow trout by disrupting peptide-MHC-I/ β 2m complex stability and interfering with key steps in antigen processing and T cell activation.

1.6 Objectives

Chapter 2:

1. Express and purify recombinant rainbow trout MHC-I and β 2-microglobulin proteins.
2. Prediction of VHSV peptide candidates with structural compatibility to rainbow trout MHC-I molecules using *in silico* tools.
3. Evaluate the MHC-I complex thermostability using *in silico* tools
4. Refold MHC-I and β 2m proteins in complex with selected VHSV peptides to form stable peptide-MHC complexes.
5. Assess unfolding behavior of MHC-I/ β 2m/peptide complexes across a temperature gradient.

Chapter 3:

1. Evaluate the effects of suboptimal temperatures on the cell surface expression of MHC-I/ β 2m in VHSV-infected cells.
2. Analyze and correlate the release of β 2m to the extracellular space to MHC-I complex recycling after infection during temperature changes.
3. Determine the processing and production of VHSV peptides by the proteasome under suboptimal temperatures.
4. Quantify the secretion of the antiviral cytokine type I interferon in VHSV-infected rainbow trout cell lines after suboptimal temperatures exposure.

Chapter 4:

1. Characterize dorsal fin cells as potential antigen presenting cells in Rainbow trout.
2. Develop an antigen presentation assay using Rainbow trout dorsal fin cells.
3. Evaluate the effect of suboptimal temperatures in the activation of T cells using Rainbow trout dorsal fin cells: analyzing cell death and cytokine secretion.

Chapter 2: Folding and stability of rainbow trout (*Oncorhynchus mykiss*) MHC-I and β 2-microglobulin complexed with VHSV IVb peptides

2.1 Overview

Antigen presentation through MHC-I molecules depends on the stable assembly and folding of a trimeric complex composed of the MHC-I heavy chain, β 2-microglobulin and an antigenic peptide. In teleost fish such as rainbow trout, little is known about the structure of this complex and its thermal stability. Understanding how temperature fluctuations affects the assembly and folding of this complex is essential for elucidating immune function in teleost species under thermal stress during viral infections. This chapter presents a bioinformatic analysis of the rainbow trout MHC-I complex, combining molecular docking, structural modeling and simulation approaches to predict how viral hemorrhagic septicemia virus (VHSV) peptides interact with MHC-I under different temperatures. In addition, *in vitro* refolding assays were performed with purified recombinant MHC-I and β 2m proteins, to further assess unfolding behavior with a temperature gradient. To accomplish these objectives, it was necessary to have all components for the final refolding of this complex: MHC-I, β 2m and VHSV-peptides. First, rainbow trout MHC-I and β 2m recombinant proteins were purified showing an efficient expression and purification after troubleshooting the methodology used. Then, VHSV-peptide candidates were chosen using proteasomal cleavage prediction tools, CD8⁺ T cells epitopes softwares, together with protein-protein docking analysis. From these data, only peptides containing hydrophobic residues in position 2 and 9 were selected, to then define structural models using AlphaFold2, which showed the interaction of these peptide candidates within the MHC-I peptide binding groove. Finally, VHSV-selected peptides were synthesized and used for the *in vitro* refolding of the MHC-I/ β 2m/VHSV-peptide complex using the purified recombinant proteins. The refolding of this complex was verified by SDS-PAGE and then used to analyze the unfolding behavior by

differential scanning fluorimetry (DSF). These findings offers both *in silico* and *in vitro* approaches to investigate the molecular structure and binding capabilities of viral pathogens like VHSV using non-model organisms such as rainbow trout. This is particularly valuable for advancing our understand of the poorly characterized antigen presentation pathway and its structural components in teleost fish under temperature fluctuations.

2.2 Introduction

The encoded MHC class I glycoprotein is a heterotrimeric complex composed of a polymorphic ~45 kDa heavy chain (HC) which is non-covalently linked to the ~12 kDa light chain, β 2-microglobulin, and an antigenic peptide (pMHC/ β 2m). The extracellular region of the heavy chain is formed by three extracellular domains (α 1, α 2 and α 3), where the α 1 and α 2 form the peptide-binding cleft or groove comprising two α helices along the sides and eight β sheets forming the platform (Figure 2.1). β 2m binds to the MHC heavy chain non-covalently through the α 3 domain, and the transmembrane domain anchors the MHC protein to the membrane (Wieczorek et al., 2017). Structural studies of the human MHC-I heterotrimeric complex showed that peptides usually ranging from 8 to 10 amino acids in length, with 9-mers being the most common, are loaded onto the peptide-binding groove of the MHC-I molecules (Sapert et al., 1991). However, there is evidence that in some cases it can bind peptides longer than 10 amino acids (Hwai-Chen Guo et al., 1992; Urbant et al., 1994). A common characteristic of these peptides, independent of their length, is the presence of hydrophobic residues at position 2 (P2) and position 9 (P9), which have a critical role in anchoring the peptide within the binding groove (Gfeller et al., 2023).

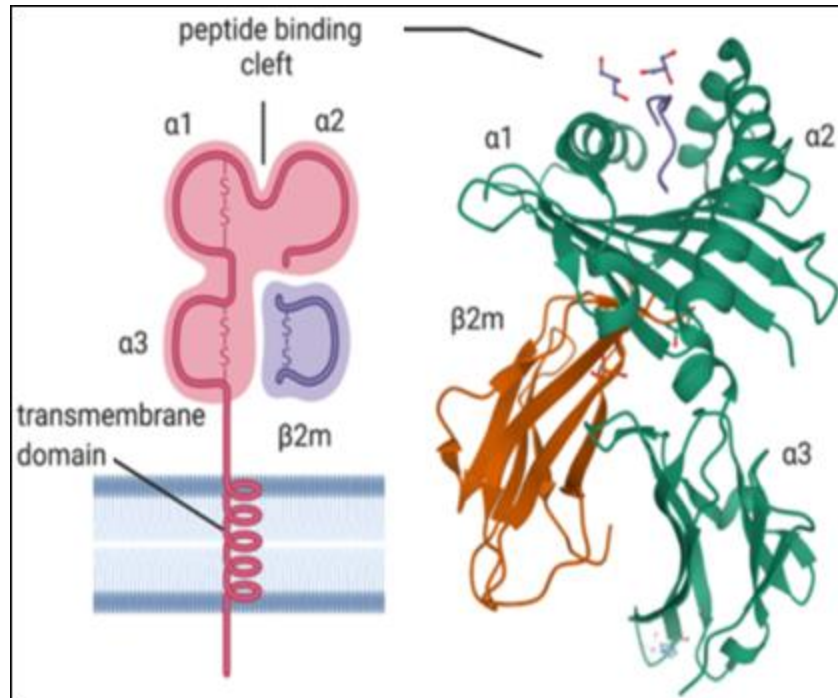


Figure 2.1. Structure of MHC-I in humans. The major histocompatibility complex class I (MHC-I) is formed by a heavy chain (HC) composed by three domains: $\alpha 1$, $\alpha 2$ and $\alpha 3$; and a light chain ($\beta 2m$). The peptide binding cleft accommodates antigenic peptides between 8-10 aa. *Image modified from Sharip & Kunz, 2020.*

The stability of the MHC-I/ $\beta 2m$ /peptide complex depends on several factors with peptide binding being the most important, where high-affinity peptides contribute to better complex stability. In addition, various ER chaperones help in the proper assembly of the complex, with $\beta 2$ -microglobulin having an essential role in maintaining MHC-I complexes stability. The conformational stability of the MHC-I directly affects their ability to be recognized by CD8⁺ T cells, therefore influencing the activation of the adaptive immune response. For this reason, several

studies have evaluated the thermostability of MHC-I complexes showing that empty MHC-I molecules, low-affinity peptides and/or MHC-I molecules not bound to $\beta 2m$, are less stable and immunogenic than MHC-I/ $\beta 2m$ complexes bound to high-affinity immunogenic peptides (Fahnestock et al., 1992; Jappe et al., 2020; Tulp & Verwoerd, 1993).

In teleost fish, MHC-I genes have high variation and different features compared to their mammalian counterparts, where their genomic organization, level of polymorphism, and association with other immune-related genes differs widely. Over the years, crystal structures of the MHC-I complex have been solved in aquatic species such as nurse shark (Wu et al., 2021) and grass carp (Chen et al., 2017). These studies have shown that in both species, these proteins have similar structural organization but there are some differences related to deletion or insertion of some residues, changing the interaction with other components of the antigen presentation pathway (Chen et al., 2017; Wu et al., 2021). Despite these advances, there is still a knowledge gap regarding how function can be affected by this genetic diversity, and particularly how peptide binding, complex stability, and immune activation are affected in species of economic importance like rainbow trout. In addition, in aquaculture, where fish are frequently exposed to fluctuating temperatures, it is especially important to understand the effects of temperature in MHC-I complex stability. Thermostability analyses are crucial to identify peptides that bind with high-affinity and serve as potential vaccines targets. Measuring thermal stability not only helps to understand peptide-MHC-I interactions but also gives insights into antigen presentation and T cell activation. Thus, the present study investigates the folding and stability of the rainbow trout MHC-I/ $\beta 2m$ complexed with viral peptides from the economically significant pathogen, viral hemorrhagic septicemia virus (VHSV). To achieve this, the chapter begins with the expression and purification

of recombinant rainbow trout MHC-I and β 2-microglobulin (β 2m) using *Escherichia coli* expression system. Then, candidate viral peptides derived from VHSV were identified through bioinformatic prediction tools. These peptides were then used to refold and assemble MHC-I/ β 2m/peptide complexes. Finally, the thermostability of the resulting complexes was analyzed by differential scanning fluorometry (DSF), providing insights into peptide binding strength and complex stability.

2.3 Materials and methods

2.3.1 Expression and purification of rainbow trout MHC-I

Recombinant constructs were synthesized by Genscript (USA) using the published rainbow trout sequence for the MHC-I UBA (GenBank entry #AF287488.1) into the expression vector pET-28b(+). The complete coding sequence for MHC-I UBA was used to synthesize the recombinant construct, except for the signal peptide and transmembrane domain. A C-terminal His-tag was placed in frame with the MHC-I gene fragment. The original rainbow trout MHC-I UBA sequence was codon optimized for expression in *E. coli*.

Competent *Escherichia coli* BL21 Star (DE3) were transformed with the recombinant plasmid pET-28b(+)-MHC-I following manufacturer's instructions. Briefly, 200 ng of plasmid DNA was added to the competent cells and incubated on ice for 20 min. The mixture was then heat-shocked at 42°C for 30 seg and then immediately returned to ice. Subsequently, SOC (Super Optimal broth with Catabolite repression) medium was added, and the cells were incubated at 37°C

for 1 h with continues shaking at 200 rpm. Transformed cells were plated onto LB (Luria-Bertani medium) agar containing kanamycin (50 mg/mL) and incubated at 37°C for 18 h. Isolated colonies were picked and used to initiate overnight cultures in LB medium. Glycerol stocks were prepared from these cultures and stored at -80°C for long-term use. Transformed *E. coli* BL21 star (DE3) with pET-28b(+)-MHC-I, were grown in ZYP-5052 autoinduction media (Studier, 2005) for 48 h at 20°C for protein expression and induced cells were frozen at -80°C until use. Induced cells were thawed and incubated with lysis buffer until fully dissolved (Table 2.1). Cells were sonicated with continuous wave ultrasound (255 kHz) in a 50s/50s pulse, for a total duration of 10 min. Cell lysates were centrifuged at 17.000 xg for 30 min at 4°C, and supernatant containing the recombinant protein was recovered. Collected recombinant protein from cell disruption process was mixed with Ni-NTA His-Bind resin, previously equilibrated with lysis buffer for 1 h at 4°C, with constant agitation. The solution was poured through a glass chromatography column (Econo-Column® Chromatography Column, Bio-Rad Laboratories) and then washed with detergent buffer (Table 2.1). Finally, lysis buffer was passed through to eliminate detergent and MHC-I recombinant protein was eluted (Table 2.1). Protein concentration was quantified at 280 nm, in a NanoDrop™ 1000 Spectrophotometer using the percent extinction coefficient (E_{280} 1% = 2.12). Recombinant protein purification steps were analyzed by SDS-PAGE.

While protein expression was successful using ZYP-5052 autoinduction media, troubleshooting was necessary to purify MHC-I recombinant protein. To do this, transformed *E. coli* BL21 star (DE3) with pET-28b(+)-MHC-I were grown in LB media until reaching an OD = 0.6 and IPTG was added at a final concentration of 1 mM and then incubated for 3 h at 37°C for protein expression. After induction, cells were centrifuged and dissolved in lysis buffer

immediately to then be sonicated following the same parameters mentioned previously. Disrupted cells were centrifuged and soluble proteins were purified using a Ni-NTA His-Bind resin as mentioned previously using buffers shown in Table 2.1. Protein concentration was quantified at 280 nm, in a NanoDrop™ 1000 Spectrophotometer using the percent extinction coefficient (E1% = 2.12). Recombinant protein purification steps were analyzed by SDS-PAGE.

Table 2.1. MHC-I purification buffers. All buffers had a pH of 8. and were stored at 4°C until use.

Buffer Name	Components
Lysis	50 mM Sodium phosphate + 0.3 M NaCl + 5 M GdnHCl + 10 mM Imidazole + 1 mM TCEP
Wash	50 mM Sodium phosphate + 0.3 M NaCl + 5 M GdnHCl + 10 mM Imidazole + 1 mM TCEP + 1% (v/v) IgePal CA-630
Elution	50 mM Sodium phosphate + 0.3 M NaCl + 5 M GdnHCl + 200 mM Imidazole + 1 mM TCEP

2.3.2 Anti-his western blot analysis

Western blots analysis were used to follow MHC-I protein purification. During both purification protocols mentioned in section 2.3.1, samples were taken in each step and run on a 12% polyacrylamide gel. Total proteins were transferred into a nitrocellulose membrane using the

Trans-Blot Turbo transfer system (Bio-Rad, USA), and complete transfer was confirmed by staining the blots with Ponceau-S (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid). Blots were incubated with 5% skim milk (w/v) in tris-buffered saline, pH 7.5, 0.1% Tween-20 (TBS-T), during 1 h at room temperature to avoid nonspecific binding. Blots were incubated with an anti-His tag antibody (1:2.000), overnight at 4°C and then incubated with an anti-mouse IgG - horseradish peroxidase conjugate secondary antibody (1:10.000, Sigma-Aldrich) for 1 h at room temperature protected from the light. The reaction was developed using the Pierce ECL Western Blotting Substrate kit (Bio-Rad, USA). Bands were visualized using the ChemiDoc XRS+ System (Bio-Rad, USA).

2.3.3 Expression and purification of rainbow trout β 2-microglobulin

The published sequence for rainbow trout β 2-microglobulin (GenBank entry #L63538.1) was utilized to synthesize recombinant construct using pET-SUMO ®Champion expression vector (Thermo Fisher) by Genscript (USA). The complete coding sequence for β 2m was used to synthesize the recombinant construct, except for the signal peptide. A N-terminal His tag and SUMO tag was placed in frame with the β 2m gene fragment. The original rainbow trout β 2m sequence was codon optimized for expression into *E. coli*.

Competent *Escherichia coli* BL21 (DE3) pLysS were transformed with the recombinant plasmid pET-SUMO- β 2m following manufacturer's instructions as previously mentioned in *section 2.3.1*. Recombinant β 2m protein expression was induced with ZYP-5052 autoinduction media for 24 h at 20°C (Studier, 2005). After induction, cells were collected by centrifugation (6000 *xg*, 10 min, 4°C) and the pellet was dissolved in lysis buffer at 4°C (Table 2.2). Cells were

then disrupted by sonication (255 kHz, 50s/50s pulse, 10 min) and centrifuged at 17.000 xg for 30 min at 4°C. Supernatant containing the recombinant $\beta 2m$ protein was mixed with equilibrated Ni-NTA His-Bind resin in lysis buffer for 1 h at 4°C. The mixture was then pass through a glass chromatography column (Econo-Column® Chromatography Column, Bio-Rad Laboratories) and then washed with detergent buffer (Table 2.2). Finally, lysis buffer was passed through to eliminate detergent and recombinant $\beta 2m$ protein was eluted. Then, the protein solution was dialyzed against an Arginine buffer using a 14 kDa MWCO dialysis tubing for 24 h at 4°C and then agaist Bicine buffer for 24 h at 4°C (Table 2.2). After dialysis, the soluble protein was incubated with the ubiquitin-like-specific protease 1 (ULP1; UniProtKB entry #Q02724) for 24 h at 4°C and then mixed with Ni-NTA His-Bind resin, previously equilibrated with Bicine buffer, for 1 h at 4°C, to separate the refolded $\beta 2m$ from SUMO-His tag. The solution was poured through a glass chromatography column, and the protein of interest was collected. Finally, the resin was washed with refolding buffer and SUMO-tag and contaminants were eluted. The protein of interest was concentrated to 20 ml using a pressure-based sample concentration (Amicon® Stirred Cells, Millipore Sigma) and then concentrated to 1 ml using a centrifugal concentrator with an ultracel membrane, 14 kDa MWCO (Amicon® Ultra-15 Centrifugal Filter Unit, Millipore Sigma).

Concentrated $\beta 2m$ was loaded into a prepacked gel filtration column Superdex™ 75 (HiLoad Superdex™ 75 pg 16/600) and 1 ml fractions were collected. Selected fractions were run on a 15% polyacrylamide gel and then concentrated using a centrifugal concentrator to 1 ml. Protein concentration was quantified at 280 nm, in a NanoDrop™ 1000 Spectrophotometer using the percent extinction coefficient ($E_{280} 1\% = 1.45$). Recombinant $\beta 2m$ protein was stored at -80°C until use.

Table 2.2. β 2-microglobulin purification buffers. All buffers had a pH of 8.5 and were stored at 4°C until use, except for Bicine that had pH 9.0.

Buffer Name	Components
Lysis	25 mM Tris-HCl + 0.3 M NaCl + 5 M GdnHCl + 10 mM Imidazole + 1 mM TCEP
Wash	25 mM Tris-HCl + 0.3 M NaCl + 5 M GdnHCl + 10 mM Imidazole + 1 mM TCEP + 1% (v/v) IgePal CA-630
Elution	25 mM Tris-HCl + 0.3 M NaCl + 5 M GdnHCl + 200 mM Imidazole + 1 mM TCEP
Arginine	25 mM Tris-HCl + 400 mM L-Arginine + 10 mM Beta-mercaptoethanol
Bicine	25 mM Bicine + 0.3 M NaCl + 1 mM TCEP
SEC (S75)	25 mM Tris-HCl + 0.3 M NaCl + 1 mM TCEP

2.3.4 Bioinformatic prediction of viral hemorrhagic septicemia peptides

To identify candidate peptides from the viral hemorrhagic septicemia virus (VHSV), the amino acid sequences from the nucleoprotein (N) and glycoprotein (G) were used (GenBank entry QKG27686.1 and QKG27689.1, respectively). A stepwise bioinformatics approach was employed to choose 9-mer peptides with better fit into the rainbow trout MHC-I UBA molecules (GenBank

entry #AF287488.1). First, the full-length amino acid sequences of VHSV N and G proteins were analyzed using NetChop 3.1, selecting the 20S proteasome mode (Nielsen et al., 2005). Cleavage predictions with scores equal or higher than 0.95 were retained as likely biologically relevant. In parallel, the same protein sequences were evaluated using Net-CTL 1.2, with the prediction model set to the MHC-I A1 supertype (Larsen et al., 2007). Peptides with affinity binding scores equal or higher than 0.5 were chosen. From the peptides predicted by both tools, those that presented hydrophobic residues in position 2 (P2) and 9 (P9) (Gfeller et al., 2023) were submitted onto the protein-protein docking software HADDOCK 2.4 (Honorato et al., 2024). Peptide-MHC-I/ β 2m complexes were scored based on HADDOCK score, van der Waals and electrostatic energies, buried surface area (BSA) and root mean square deviation (RMSD). The two-best performing peptides from each VHSV protein were selected for structural modeling. To further assess peptide compatibility, the structures of the rainbow trout MHC-I heavy chain with β 2-microglobulin and the selected peptides were predicted using AlphaFold2 via ColabFold platform (Jumper et al., 2021). Molecular graphics and analysis were performed with UCSF ChimeraX (Meng et al., 2023). The selected peptides were synthesized by Genscript (USA).

2.3.5 Structural flexibility analysis of MHC-I/ β 2m/VHSV-predicted peptides

The structural flexibility of rainbow trout MHC-I/ β 2m/VHSV-peptide complexes was evaluated using CABS-flex 2.0. Predicted structural models of MHC-I/ β 2m complexed with VHSV predicted peptides from protein N and G (*section 2.3.4*) was uploaded to CABS-flex 2.0 and run under default simulation parameters, except for the simulation temperature, which was adjusted to three different values: 0.5, 1.5 and 2.5. These values correspond to low, moderate and high temperatures. For each simulation, CABS-flex 2.0 generated models and calculated root mean

square fluctuations (RMSF) values across residues for each protein and/or peptide. The RMSF was plotted to visualize residue-specific flexibility under each temperature.

2.3.6 Folding of MHC-I/ β 2m complexed with VHSV predicted peptides

Synthetically prepared VHSV peptides were dissolved in DMSO to a final concentration of 1M and store at -80°C until further use. The MHC-I UBA and β 2m recombinant proteins (section 2.3.1 and 2.3.3, respectively), were mixed with the synthetic VHSV peptides in a molar ratio of 1:2:10 (MHC-I: β 2m:peptide) and refolded by dilution in refolding buffer (100 mM Sodium phosphate; pH 8.0, 400 mM L-Arginine, 5 mM GSH, 0.5 mM GSSH, 1 mM EDTA) and incubated for 48 h at 4°C. After incubation, the refolding mixture was centrifuged at 14.000 xg for 30 min at 4°C and the soluble portion was recovered. Half of the soluble proteins were precipitated by incubating with sodium deoxycholate (1.5 mg/mL) and 72% TCA for 10 min on ice. Precipitated proteins were centrifuged at 14.000 xg for 15 min at 4°C and then washed with cold acetone. Soluble and precipitated proteins were analyzed by SDS-PAGE.

2.3.7 Differential scanning fluorometry (DSF)

Folded MHC-I/ β 2m/VHSV-peptide complexes were mixed with the SYPRO Orange Protein gel stain at a final concentration of 10X in a total volume of 75 μ l. Samples were then loaded in triplicate in MicroAmp® Optical 8-Cap strips with a final volume of 20 μ l and then run in QS6 RT-PCR instrument. The temperature scan rate was set at 0.05°C/sec ranging from 10°C to 90°C. Refolding buffer, MHC-I, β 2m and MHC-I/ β 2m were loaded as controls together with the refolded complexes.

2.4 Results

2.4.1 Protein expression and purification of rainbow trout MHC-I UBA

To obtain denatured MHC-I proteins, *E. coli* BL21 Star (DE3) were transformed with the recombinant vector pET-28b(+) containing the coding sequence for rainbow trout MHC-I UBA. After protein induction, an 12% SDS-PAGE was run to follow the purification steps of MHC-I. As mentioned in *section 2.3.1*, two expression methods were used to then purify the recombinant protein. In figure 2.2.A, the resulting gel is shown using autoinduction media ZYP-5052 together with the purification steps using a Ni-NTA resin. A band of the expected molecular weight of ~35 kDa was observed after protein induction. In addition, a strong band was observed in all purification steps, except for the eluted proteins, at a molecular weight of ~35 kDa (Figure 2.2.A). Since proteins were not eluted, a western blot analysis using an anti-His tag antibody was performed to confirm the presence of the tag. As observed in Figure 2.2.B, a ~ 35 kDa band is observed after protein induction and total protein sample before sonication, however, no bands were observed in the following steps during the protein purification (Figure 2.2.B).

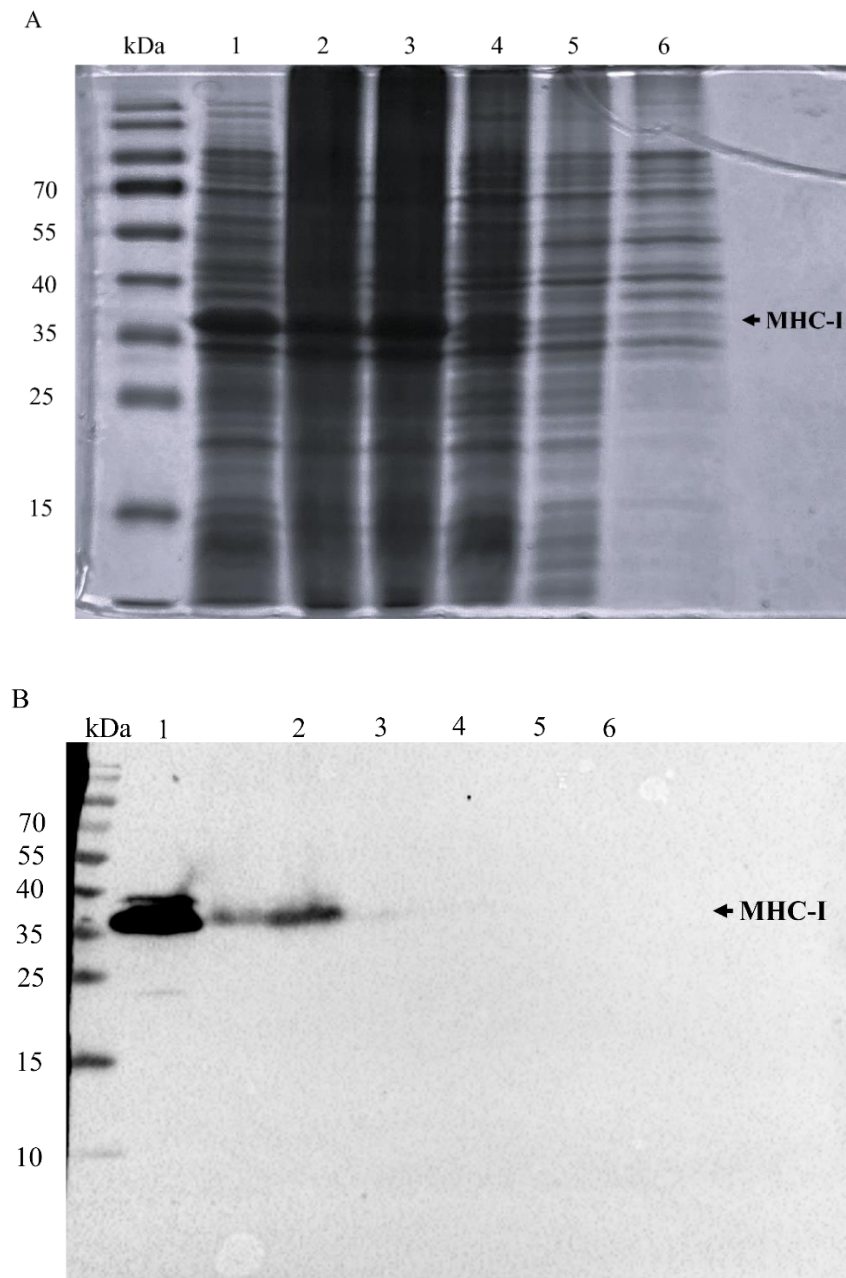


Figure 2.2. Protein purification of MHC-I using autoinduction medium. SDS-PAGE 12% gels were used to analyze MHC-I protein expression and purification by autoinduction medium. (A) Lane identities include induced protein (lane 1), total lysed protein (lane 2), post-sonication soluble protein (lane 3), protein purification flow through (lane 4), wash (lane 5) and eluted protein (lane

6). (B) Western blot analysis using an anti-His tag antibody of MHC-I protein purification using autoinduction medium. Lanes were loaded as described for figure 2.2.A.

After the negative result using autoinduction ZYP-5052 media, the protocol was changed to induce the recombinant protein using IPTG. Induced proteins were dissolved in lysis buffer, then sonicated and soluble proteins were maintained for 4 h at room temperature to follow protein degradation (Figure 2.3.A). The presence of His-tag was evaluated by western blot as mentioned before (Figure 2.3.B). As shown in Figure 2.3, a ~ 35 kDa band was observed after IPTG induction, which was also observed after 4 h in lysis buffer. In addition, positive bands of ~ 35 kDa were observed in the developed blots using an anti-His tag antibody confirming the presence of the tag (Figure 2.3.B). After confirming the presence of His tag, MHC-I was purified as mentioned previously and protein concentration of eluted protein was quantified resulting in a yield of 3.25 mg per liter of culture. Protein was stored in elution buffer at 4°C until the refolding steps (Figure 2.4).

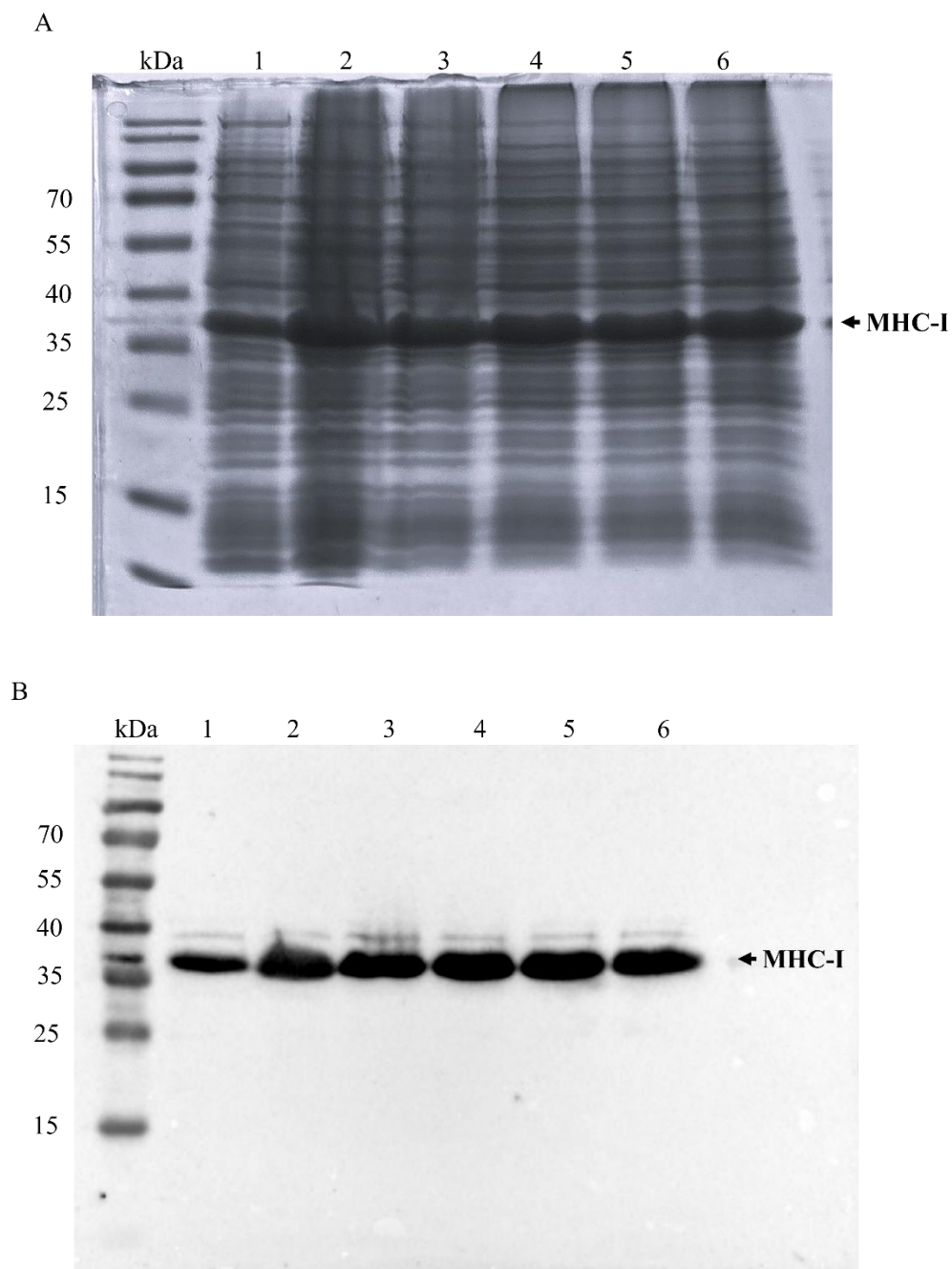


Figure 2.3. Protein purification and stability of MHC-I using IPTG. SDS-PAGE 12% gels were used to analyze MHC-I protein expression and purification using IPTG. (A) Protein induction and stability was analyzed. Lane identities include uninduced protein (lane 1), total induced protein

(lane 2) total lysed protein (lane 3), post-sonication soluble protein (lane 4), protein in lysis buffer for 1 h at room temperature (lane 5) and protein in lysis buffer for 4 h at room temperature (lane 6). (B) Western blot analysis using an anti-His tag antibody of MHC-I protein purification and stability using IPTG. Lanes were loaded as described for figure 2.3.A.

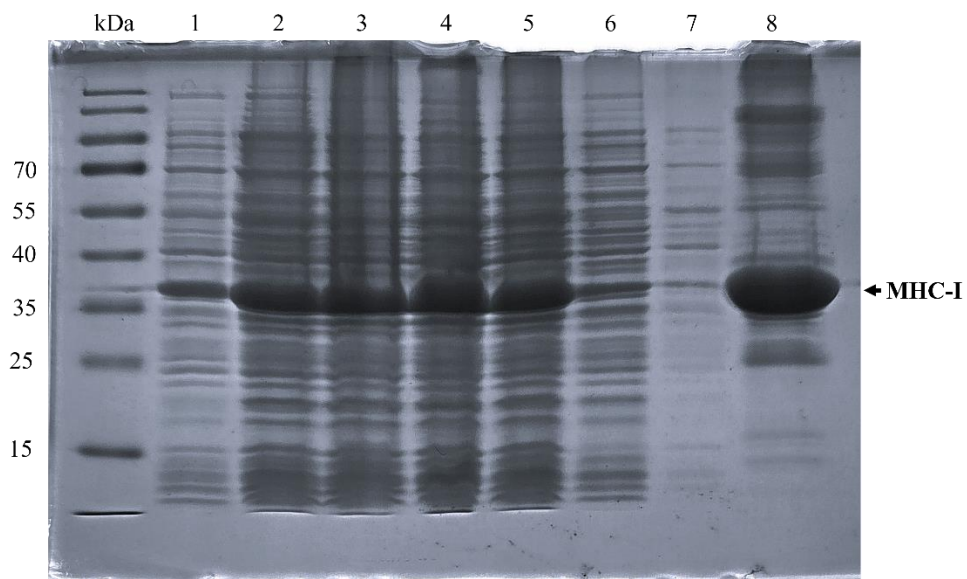
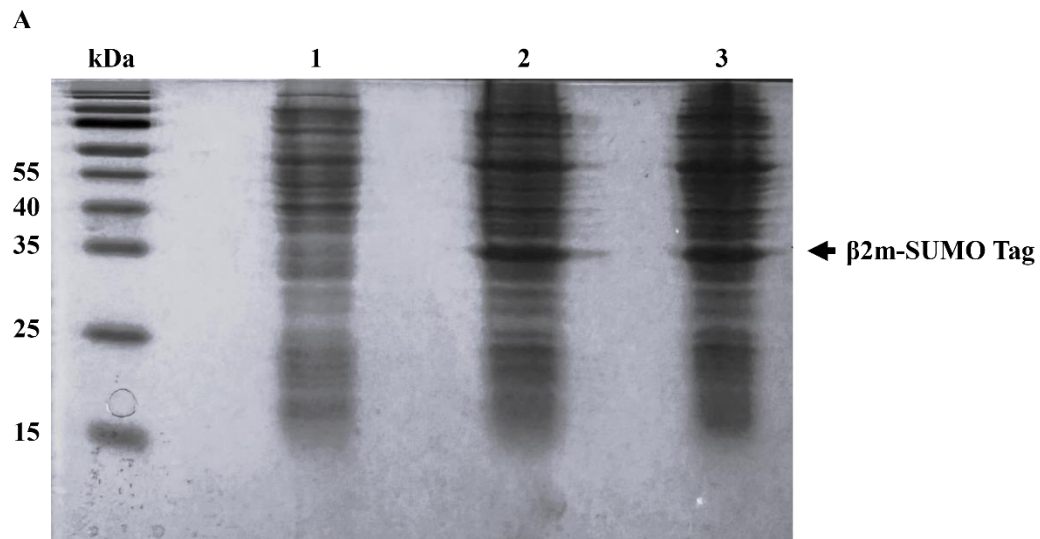


Figure 2.4. Protein purification of MHC-I. Protein purification of MHC-I after confirming presence of His tag using IPTG for protein induction. Lane identities include uninduced protein (lane 1), induced protein (lane 2), total lysed protein (lane 3), post-sonication mixture (lane 4), post-sonication soluble protein (lane 5), protein purification flow through (lane 6), wash (lane 7) and eluted protein (lane 8).

2.4.2 Expression, purification and refolding of rainbow trout β 2-microglobulin

E. coli BL21 (DE3) pLysS were transformed with the recombinant vector pET-SUMO®Champion- β 2m to express and purify rainbow trout β 2-microglobulin protein. The expression of this protein was induced by using autoinduction media, resulting in a strong band of ~24 kDa (Figure 2.5.A). Bacterial cells were disrupted by sonication and the protein of interest was purified by affinity chromatography using a Ni-NTA His-Bind resin. Denatured β 2m was then refolded in Arginine and Bicine buffer and refolded proteins were incubated with the ULP1-protease to cleave the SUMO-His-Tag. Both tags and contaminants were separated from the recombinant β 2m protein by affinity chromatography to obtain the final purified recombinant protein. Aggregates were separated from the folded β 2m by size-exclusion chromatography, resulting in a pure band of ~11 kDa (Figure 2.5.B). Proteins were stored at -80°C to further use for *in vitro* assays.



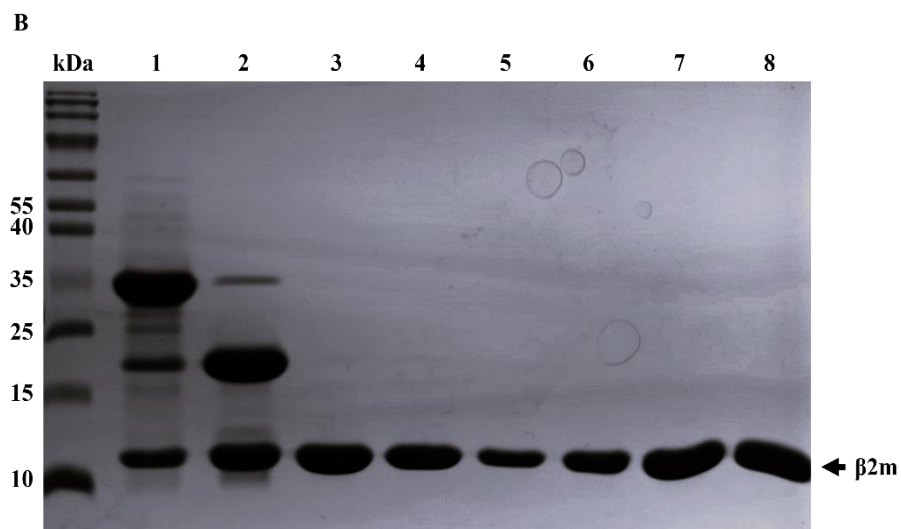


Figure 2.5. Protein expression and purification of β 2-microglobulin. SDS-PAGE 15% gels were used to analyze β 2m protein expression by autoinduction medium. (A) Lane identities include: uninduced protein (lane 1), total induced protein at 24 h (lane 2) and total induced protein at 48 h (lane 3). (B) For protein purification, gels were loaded in the next order: total eluted protein (lane 1), SUMO tag cleavage (lane 2), size-exclusion chromatography S75 fractions (lane 3-8).

2.4.3 Bioinformatic prediction of VHSV peptide candidates

To refold the recombinant MHC-I/ β 2m proteins with VHSV peptides, it was necessary to predict and choose the best-fitting viral peptide candidates. This was performed using different bioinformatic prediction tools to analyze the binding of VHSV protein N and G peptides to the peptide binding groove of MHC-I UBA. Since viral peptides are generally produced by the ubiquitin-proteasome system, the proteasomal cleavage software NetChop 3.1 was utilized to

predict the cleavage sites for both N and G protein amino acid sequences, and scores higher than 0.95 were chosen (Table 2.3.1 and 2.3.2, respectively).

Table 2.3.1. NetChop 3.1 analysis of VHSV N protein. 20S proteasomal cleavage position, amino acid and NetChop 3.1 score are shown for the amino acid sequence of VHSV N protein (GenBank entry # QKG27686.1).

Position	Amino acid	Score	Position	Amino acid	Score
7	A	0.97428	232	A	0.961102
9	F	0.954533	244	L	0.952438
69	L	0.976578	262	L	0.971262
88	L	0.957669	274	E	0.964859
90	D	0.980337	282	R	0.982957
121	A	0.966384	286	E	0.980816
124	L	0.965904	304	L	0.963916
135	C	0.959762	326	E	0.972574
179	L	0.970238	331	F	0.964172
194	Y	0.967546	341	D	0.95383
225	M	0.9582	377	R	0.984313
7	A	0.97428	232	A	0.961102
9	F	0.954533	244	L	0.952438
69	L	0.976578	262	L	0.971262

Table 2.3.2. NetChop 3.1 analysis of VHSV G protein. 20S proteasomal cleavage position, amino acid and NetChop 3.1 score are shown for the amino acid sequence of VHSV G protein (GenBank entry #QKG27689.1).

Position	Amino acid	Score	Position	Amino acid	Score
8	L	0.964772	249	M	0.96718
31	I	0.951494	253	V	0.968439
52	D	0.950475	255	F	0.964772
62	L	0.970044	302	L	0.982774
68	F	0.968501	309	M	0.959825
82	I	0.980876	358	E	0.952674
97	R	0.978922	374	Y	0.959943
106	Y	0.962404	384	W	0.965421
157	D	0.950863	412	Q	0.957633
173	V	0.977433	416	D	0.966992
180	R	0.987098	422	Q	0.981954
204	V	0.964283	423	R	0.97621
228	R	0.981809	436	V	0.975078
247	L	0.977437	458	F	0.976055

In parallel, the CD8⁺ T cells epitope prediction software Net-CTL 1.2 was used to choose VHSV peptides for the N and G protein. The sequences with better affinity, proteasome cleavage and transport through the TAP transporter were chosen (Table 2.4.1 and 2.4.2, respectively). From these peptides, only the ones with hydrophobic residues in position 2 (P2) and 9 (P9) were selected to continue with protein docking analysis using the HADDOCK 2.4 software.

Table 2.4.1. Net-CTL 1.2 analysis of VHSV N protein. CD8⁺ T cell epitopes prediction for the amino acid sequence of VHSV N protein (GenBank entry # QKG27686.1). Predicted sequences, affinity, cleavage and TAP transporter scores are shown.

Sequence	Affinity	Cleavage	TAP
QALSVLLSY	0.6619	0.9595	3.0550
TMIGLFTQA	0.7767	0.1879	0.2750
MIGLFTQAA	2.4221	0.8691	3.0070
YSVDKMIKY	2.1523	0.9476	3.1240
MSRMLGESY	1.1718	0.4268	3.0970
LNDNSKISY	0.8723	0.9562	2.5700
NADNLMDIV	1.2932	0.9788	2.9110
VAEVLVDKY	1.3670	0.8407	2.9430
KSDPKARNF	0.6958	0.9054	2.6600

Table 2.4.2. Net-CTL 1.2 analysis of VHSV G protein. CD8⁺ T cells epitopes prediction for the amino acid sequence of VHSV N protein (GenBank entry # QKG27689.1). Predicted sequences, affinity, cleavage and TAP transporter scores are shown.

Sequence	Affinity	Cleavage	TAP
VSAVARGHY	2.0580	0.3498	3.0980
NVHKDITHY	0.7025	0.9760	2.9910
SIDLYSRKF	0.5434	0.8449	2.5870
LTMACTVTF	0.5387	0.8325	2.5520
KTDLGDLIK	1.0254	0.8890	0.3830
ATDDFSYLN	1.2601	0.0823	-1.3990
HSDITASGK	0.9968	0.2544	0.3920
YEAVVSINY	0.6157	0.9650	2.6470
SINYNSAQY	0.9895	0.9510	3.0590
TVTFCGAEW	2.0798	0.7653	3.2620
NTDGYDGMI	1.5849	0.7675	0.5150
LVQRNLVEI	1.7960	0.8604	2.4480

The docking analysis showed different parameters and based on these parameters the best fitting peptides for each VHSV protein were selected. Peptides with favorable docking (haddock score), steric (van der Waals energy) and charge-based interactions (electrostatic energy) were selected, in addition to stronger binding to the peptide-binding groove of MHC-I (buried surface area), and more reliable docking model (root mean square deviation for VHSV protein N and G (Figure 2.6.1 and 2.6.2. respectively).

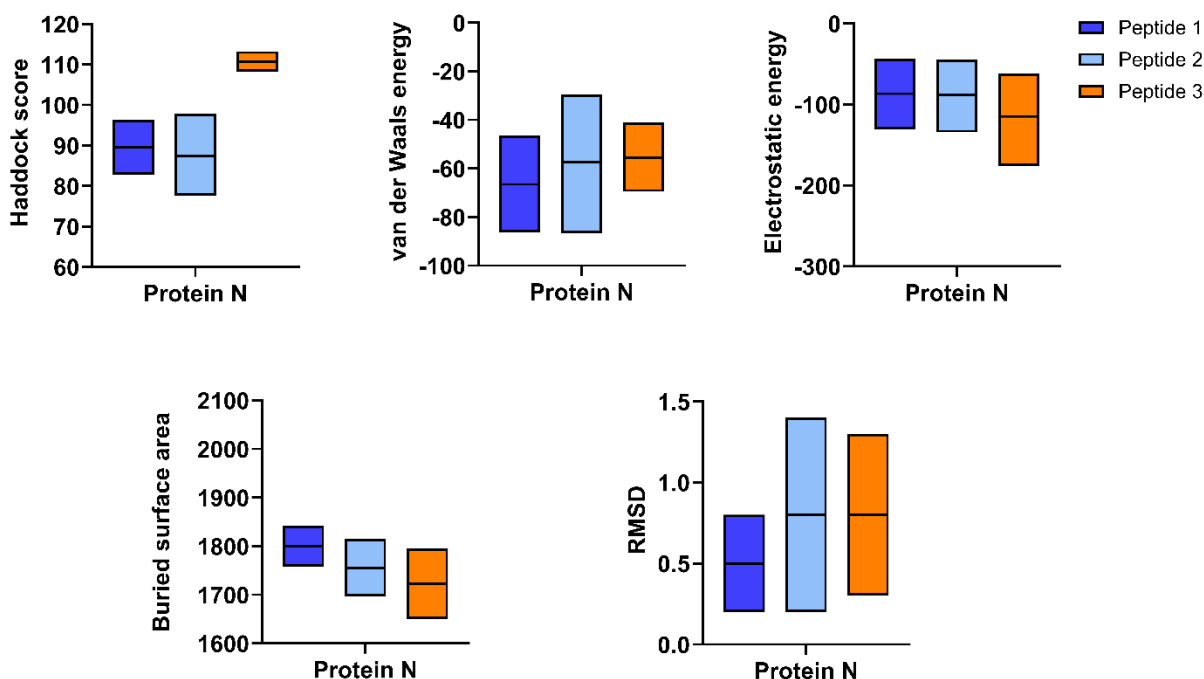


Figure 2.6.1. Protein docking analysis using HADDOCK 2.4 of VHSV protein N. The selected peptides from NetChop 3.1 and Net-CTL 1.2 (Table 2.3 and 2.4) were used to perform docking-docking analysis with the MHC-I UBA predicted structure with HADDOCK 2.4 software. Haddock score, van der Waals and electrostatic energies, buried surface area and root mean square

deviation is shown. The sequences analyzed were: TMIGLFTQA (peptide 1), MIGLFTQAA (peptide 2) and NADNLMDIV (peptide 3).

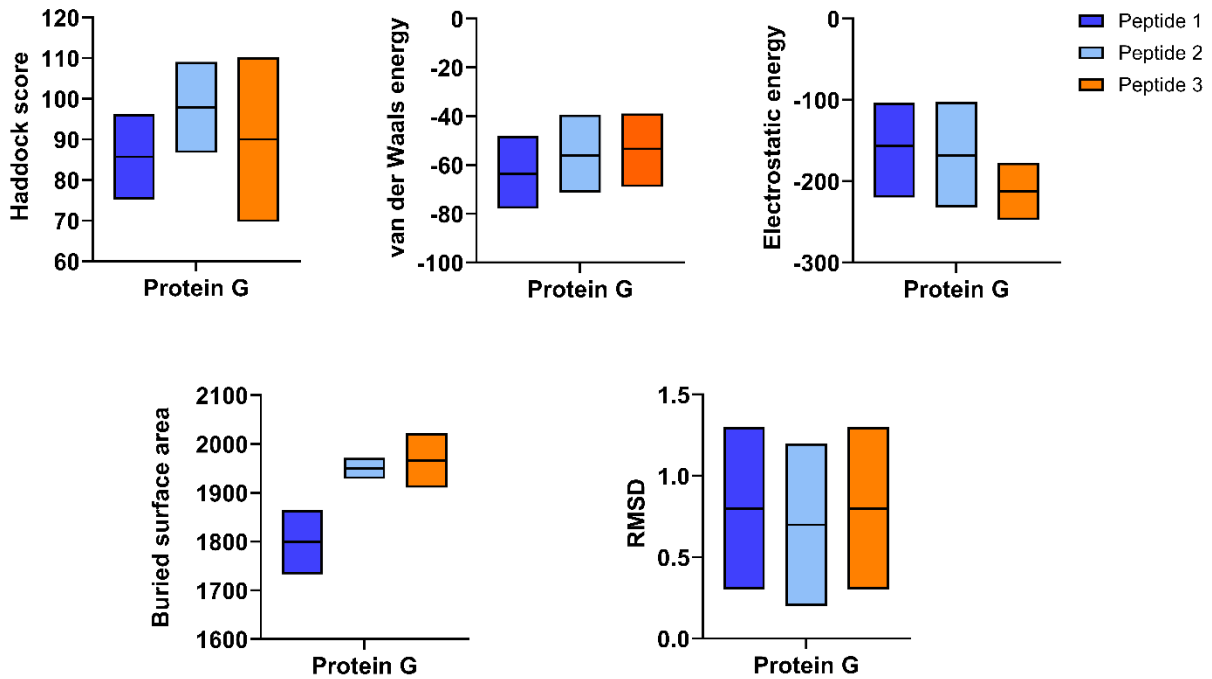


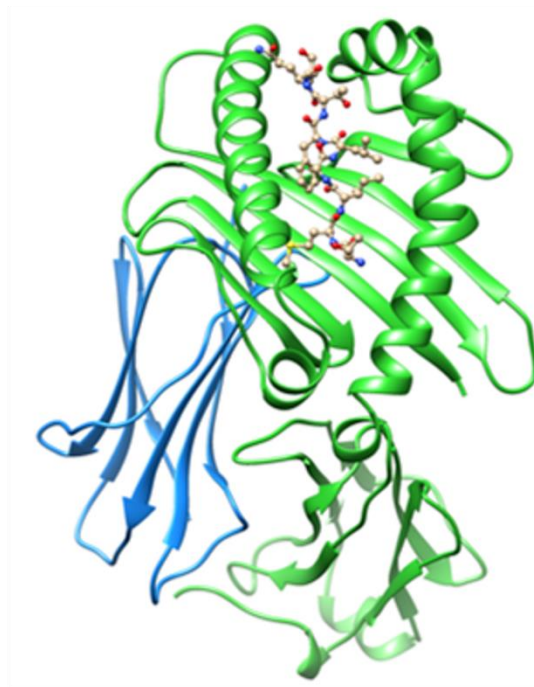
Figure 2.6.2. Protein docking analysis using HADDOCK 2.4 of VHSV protein G. The selected peptides from NetChop 3.1 and Net-CTL 1.2 (Table 2.3 and 2.4) were used to perform docking-docking analysis with the MHC-I UBA predicted structure with HADDOCK 2.4 software. Haddock score, van der Waals and electrostatic energies, buried surface area and root mean square deviation is shown. The sequences analyzed were: TVTF CGAEW (peptide 1), SIDLYSRKF (peptide 2) and LVQRNLVEI (peptide 3).

With the selected peptides for each VHSV protein, structure prediction analysis of the rainbow trout MHC-I/ β 2m/VHSV-peptide complexes were performed with AlphaFold2 via

ColabFold platform. Predicted structures were evaluated in USFC ChimeraX, as shown in Figure 2.7, where the binding of $\beta 2m$ to the $\alpha 3$ domain of MHC-I molecule can be seen as well as the fit of the chosen VHSV peptides, for protein N and G, into the peptide-binding groove formed by the $\alpha 1$ - $\alpha 2$ domain of MHC-I UBA (Figure 2.7A and B, respectively).

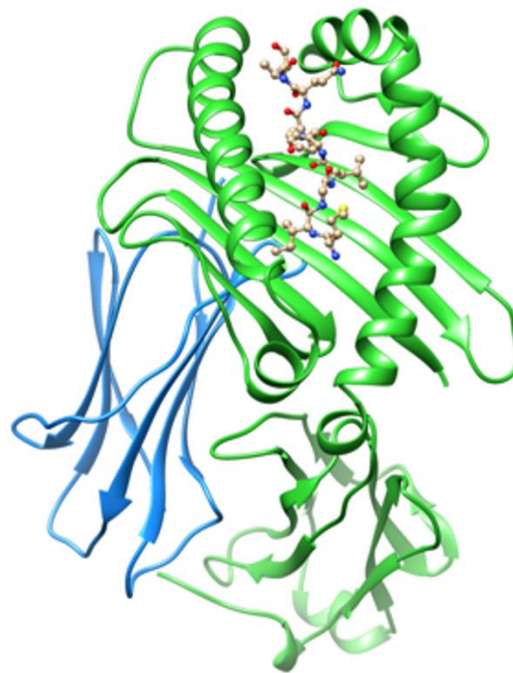
A

MHC-I/ $\beta 2m$ /peptide
Protein N peptide 1



VHSV Protein N peptide 1
TMIGLFTQA

MHC-I/ $\beta 2m$ /peptide
Protein N peptide 2



VHSV Protein N peptide 2
MIGLFTQAA

B

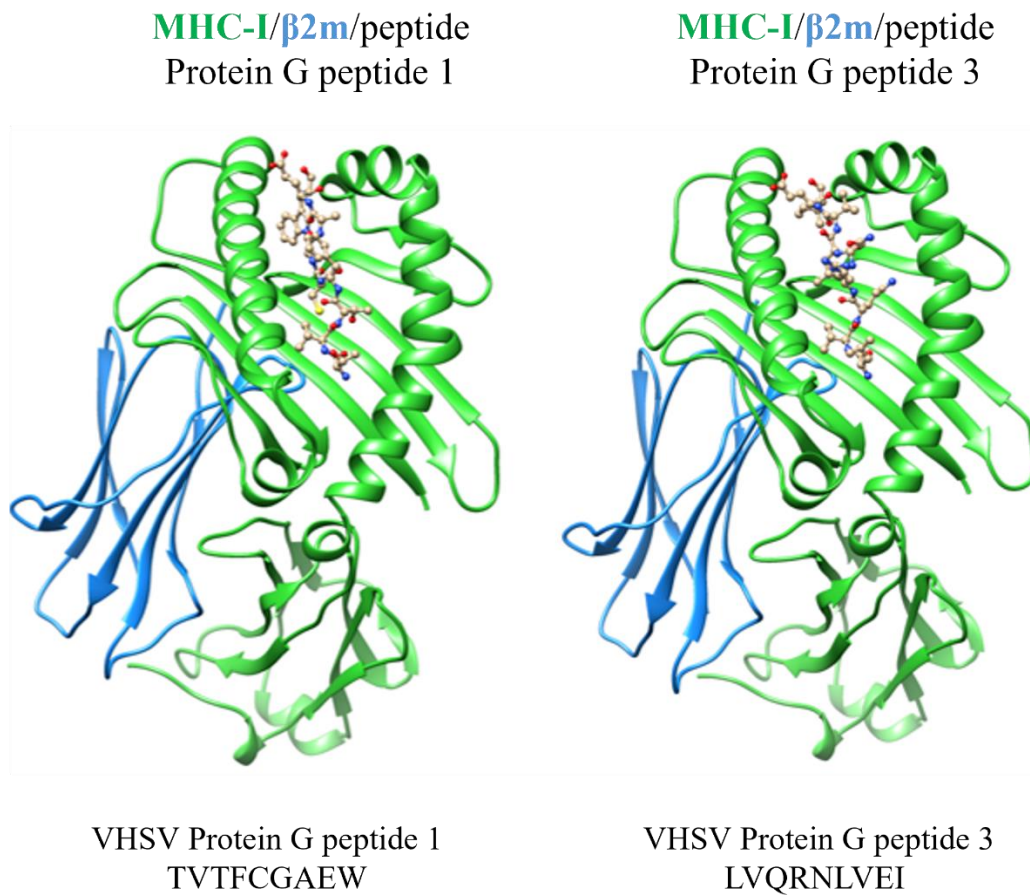


Figure 2.7. Structure prediction of rainbow trout MHC-I UBA/ β 2m/VHSV-predicted peptides.

Molecular structure of the rainbow trout MHC-I UBA (green) bound to β 2-microglobulin (blue) and complexes with chosen peptides for VHSV (khaki) protein N (A) and G (B) predicted by AlphaFold2. The predicted model had AlphaFold confidence scores ranging from 55.4 to 98.9 (pLDTT > 81; Protein N1), 50 to 98.9 (pLDTT > 82; Protein N2), 48.4 – 98.9 (pLDTT > 81; Protein G1), 48.8 to 98.9 (pLDTT > 80; Protein G3)..

2.4.4 Temperature-dependent structural flexibility of MHC-I/ β 2m/VHSV-peptide complexes

The average residue flexibility of MHC-I/ β 2m complexed with VHSV predicted peptides was evaluated using the CABSflex 2.0 server under three temperature parameters: low, moderate and high. The root mean square fluctuation (RMSF) values obtained for the MHC-I chain showed a minimum fluctuation at low temperature (0.5) (Figure 2.8). At moderate temperature (1.5), a higher flexibility was observed compared to lower temperatures across multiple regions with all peptides tested. The highest overall fluctuations were observed at higher temperatures (2.5), where elevated RMSF values were shown indicating greater conformational mobility (Figure 2.8).

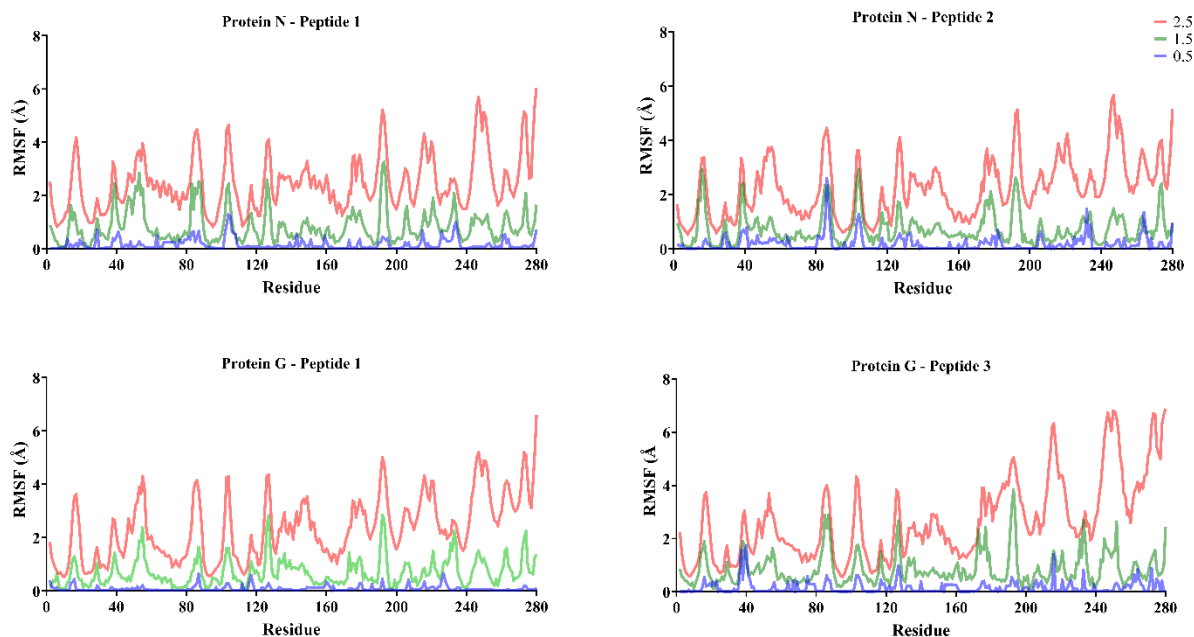


Figure 2.8. Structural flexibility of MHC-I chain at different temperatures. Predicted models of MHC-I/ β 2m complexed with VHSV-peptides were uploaded to CABS-flex 2.0 and analyzed at

low (blue), moderate (green) and high (red) temperatures. Root mean square fluctuations (RMSF) were plotted. VHSV peptides were used to predict the structure flexibility of MHC-I (Protein N – peptide 1: TMIGLFTQA, protein N – peptide 2: MIGLFTQAA, protein G – peptide 1: TVTFCGAEW and protein G – peptide 3: LVQRNLVEI).

The structural flexibility of $\beta 2m$ remained relatively stable when simulated at low temperatures, showing low fluctuations, as well with the higher temperatures, where similar profiles were observed across all peptide-bound complexes. However, moderate temperatures, revealed variation among different peptides, with distinct RMSF peaks observed at different residue positions (Figure 2.9).

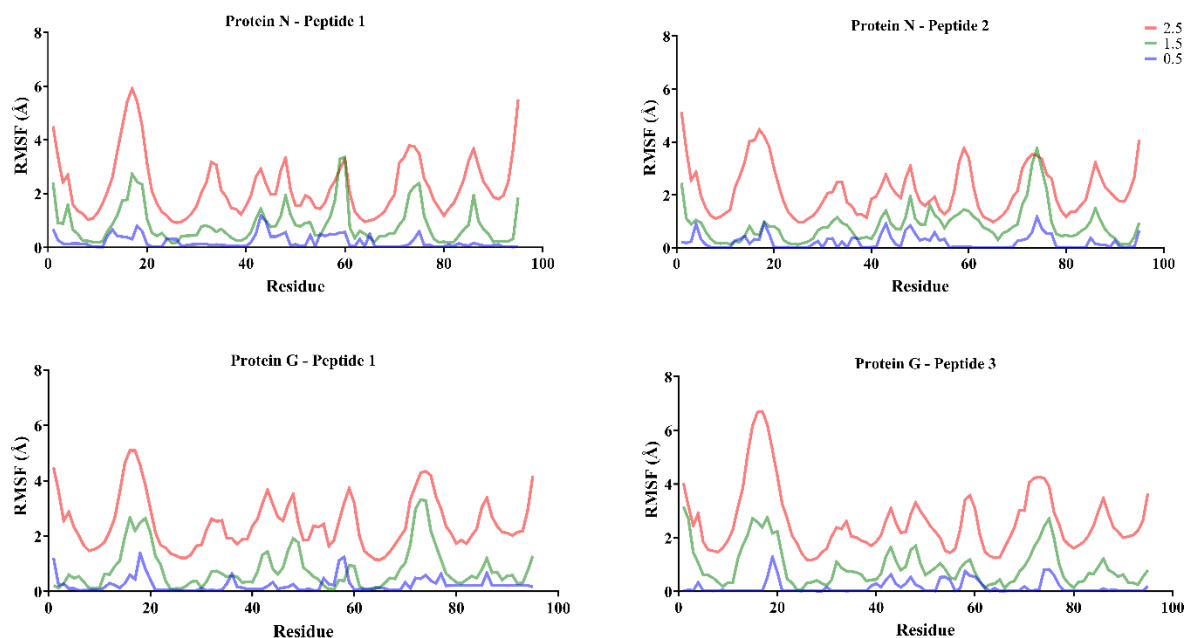


Figure 2.9. Structural flexibility of $\beta 2m$ chain at different temperatures. Predicted models of MHC-I/ $\beta 2m$ complexed with VHSV-peptides were uploaded to CABS-flex 2.0 and analyzed at low (blue), moderate (green) and high (red) temperatures. Root mean square fluctuations (RMSF) were plotted. VHSV peptides were used to predict the structure flexibility of $\beta 2m$ (Protein N – peptide 1: TMIGLFTQA, protein N – peptide 2: MIGLFTQAA, protein G – peptide 1: TVTFCGAEW and protein G – peptide 3: LVQRNLVEI).

The structural flexibility of VHSV peptides complexes with MHC-I/ $\beta 2m$ was evaluated and showed limited fluctuations at low temperature settings (0.5), with exception of Protein G – peptide 3, which showed elevated RMSF values at residues 6 – 9 (Figure 2.10). At moderate temperature (1.5), all peptides showed relatively stable RMSF values remaining below 2 Å. However, at the highest temperature (2.5), flexibility increased compared to the other temperatures tested, especially at the C-terminal region, with most pronounced fluctuations mainly in Protein G – peptide 3 (Figure 2.10).

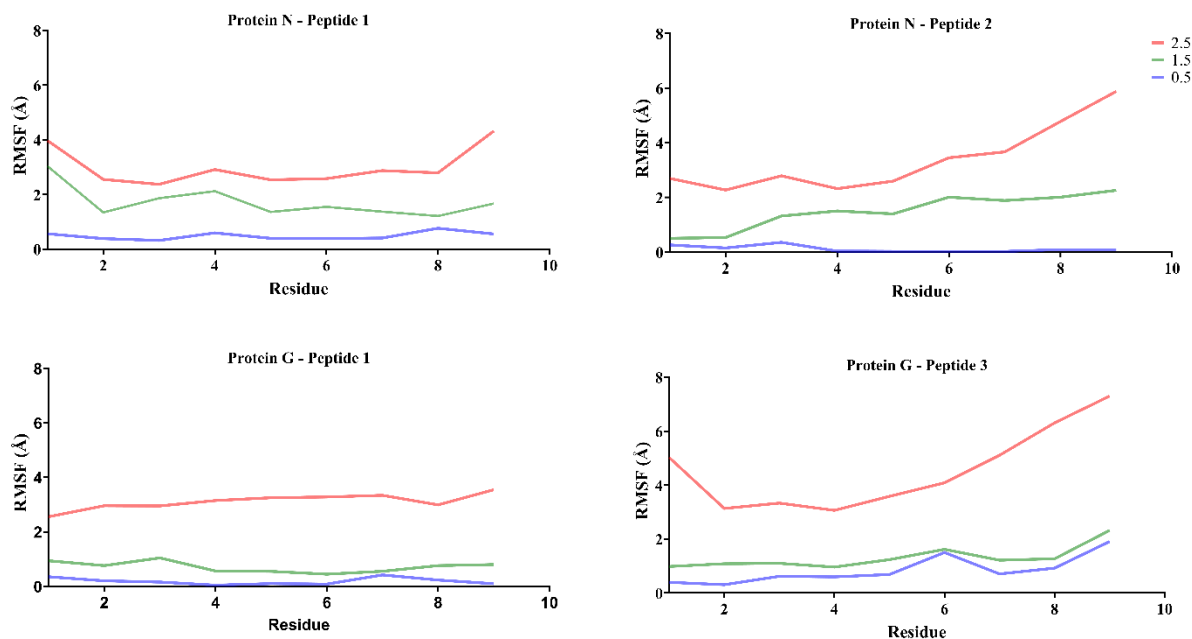


Figure 2.10. Structural flexibility of VHSV peptides at different temperatures. Predicted models of MHC-I/β2m complexed with VHSV-peptides were uploaded to CABS-flex 2.0 and analyzed at low (blue), moderate (green) and high (red) temperatures. Root mean square fluctuations (RMSF) were plotted. The structure flexibility of VHSV peptides was evaluated when complexed with MHC-I/β2m (Protein N – peptide 1: TMIGLFTQA, protein N – peptide 2: MIGLFTQAA, protein G – peptide 1: TVTF CGAEW and protein G – peptide 3: LVQRNLVEI).

2.4.5 Folding of MHC-I/β2m/VHSV-peptides complex

Once VHSV peptides were selected, they were synthesized by Genscript (USA) and subsequently used to refold with the purified MHC-I/β2m proteins mentioned in section 2.3.1 and 2.3.3. As observed in Figure 2.11, when precipitated with TCA the soluble fraction of the *in vitro*

refolding assay contained both MHC-I (~ 35 kDa) and β 2m (~ 11 kDa) proteins after refolding with all chosen VHSV peptides. However, without precipitation, a band corresponding to MHC-I was only observed when Protein G – peptide 1 (TVTFCGAEW) was bound (Figure 2.11).

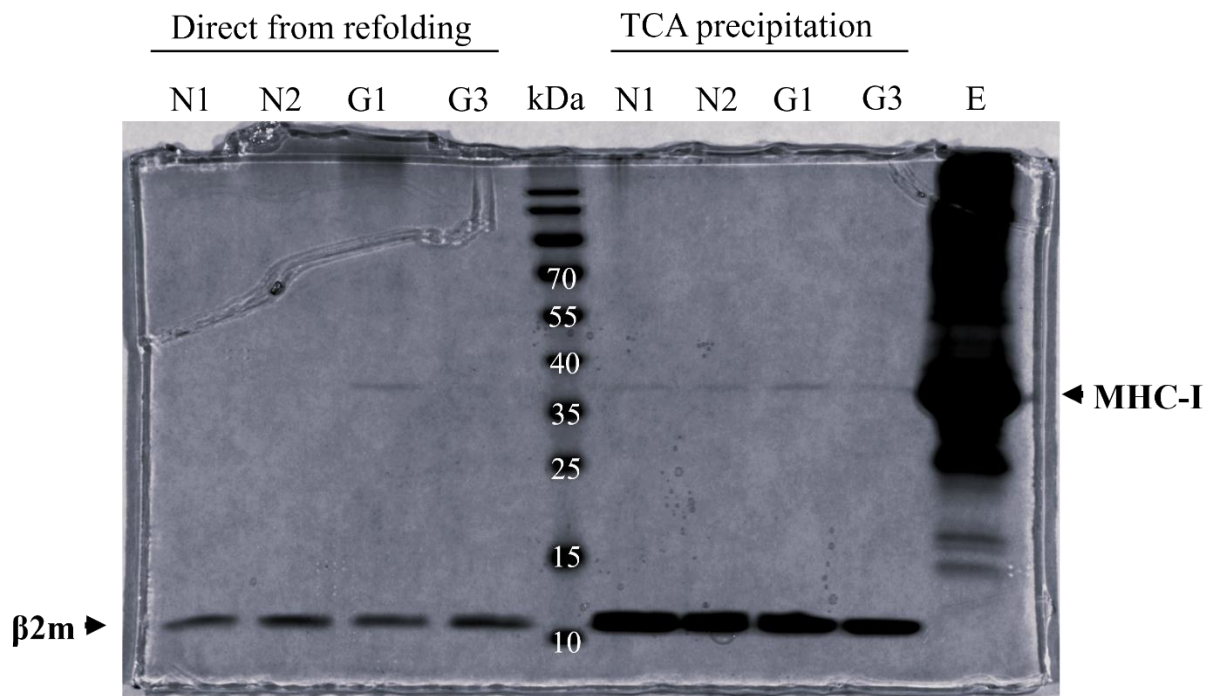


Figure 2.11. *In vitro* refolding of MHC-I/ β 2m complexed with VHSV-predicted peptides.

Recombinant MHC-I and β 2m proteins were refolded with predicted VHSV peptides in a molar ratio of 1:2:10, respectively, and analyzed by SDS-PAGE. Lanes are non-precipitated soluble proteins (left side of the protein ladder) and precipitated soluble proteins (right side of the protein ladder). Protein N – peptide 1 (N1: TMIGLFTQA), Protein N – peptide 2 (N2: MIGLFTQAA), Protein G – peptide 1 (G1: TVTFCGAEW) and Protein G – peptide 3 (G3: LVQRNLVEI), were

used to refold the complex. A positive control of eluted MHC-I derived from the purification process was used (last lane).

2.4.6 Unfolding behavior of MHC-I/ β 2m/VHSV-peptides

The thermostability of the folded MHC-I/ β 2m/VHSV-peptides was analyzed by differential scanning fluorometry (DSF) to determine whether VHSV peptide binding stabilized the complex. However, no increase in fluorescence signal was detected for any of the refolded complexes throughout the temperature gradient. The unfolding profiles for all conditions, were comparable to the refolding buffer alone control.

2.5 Discussion

The folding and assembly of MHC-I molecules with β 2m and antigenic peptides is a crucial step during antigen processing and presentation and further activation of adaptive immunity. Unfortunately, little is known about this process in rainbow trout, and less on how temperature fluctuations could affect the proper folding of these molecules. This chapter described the purification of recombinant rainbow trout MHC-I and β 2-microglobulin (β 2m) proteins, along with bioinformatics prediction and modeling of VHSV-derived peptides candidates for *in vitro* refolding and molecular flexibility at different temperatures with the MHC-I/ β 2m complex.

One of the many challenges in this chapter was the expression and purification of the recombinant MHC-I protein. Initially, protein expression was performed using ZYP-5052 autoinduction media (Studier, 2005) which resulted in successful protein expression, as shown by a clear ~35 kDa band after 48 hours of induction (Figure 2.2.A). However, the protein failed to

bind to the Ni-NTA resin during purification as observed by the absence of a clear band in the eluted proteins samples (Figure 2.2.A). One possibility to this problem was that the His-tag was inaccessible or that the MHC-I protein was aggregated, leading to poor or inexistent binding to the Ni-NTA resin. This is very common when expressing eukaryotic proteins in *E. coli* systems (Peternel & Komel, 2011). To investigate this, western blotting was performed using an anti-His tag antibody, which resulted in a strong signal showed as a single band at ~35 kDa in the total lysate after induction, which was significantly reduced in the dissolved pellet and completely lost after sonication and subsequent steps, suggesting the proteolytic degradation of the His-tag (Figure 2.2.B). Since that autoinduction involves long incubation, in this case 48 h, and that cell pellets are stored at -80°C before lysis, it is possible that prolonged exposure to proteases during or after expression led to partial or complete cleavage of the His-tag. In addition, the process of slowly thawing frozen pellets in lysis buffer may have helped to the proteases to cleave the exposed His-tag. Researchers have shown that several *E. coli* proteases are capable of cleaving proteins at or near the C-terminal region, where His-tags are typically placed, as in our case (Lykkemark et al., 2014).

To solve the problems encountered with autoinduction, IPTG expression strategy was used using a shorter induction time (3 hours at 37°C). During this test, induced cell pellets were immediately dissolved in lysis buffer, instead of store them at -80°C as usual. The latter probably reduced the proteolytic degradation of the His-tag. Western blot analysis using an anti-His tag antibody confirmed the presence of the tag both after induction and during purification, confirming that delayed processing of the samples increased the chance of proteolysis (Figure 2.3). After confirming the integrity of the His-tag, the MHC-I protein was purified under denaturing

conditions using guanidine hydrochloride, since the proper folding with $\beta 2m$ and viral peptides requires the fully denatured MHC-I protein before *in vitro* refolding (Z. Chen et al., 2017; Garboczi et al., 1992). Samples were taken during all purification steps and by running an SDS-PAGE it was confirmed a strong band at the expected molecular weight (~ 35 kDa) in the eluted proteins (Figure 2.4).

Contrasting the MHC-I protein purification, the expression and purification of $\beta 2m$ was more efficient and easier to troubleshoot. In this case, the pET-SUMO vector was chosen to help in expression and purification of $\beta 2m$. Usually, the SUMO tag is used to increase the protein solubility and stability acting as a protein chaperone (Marblestone et al., 2006), however, despite the use of this tag, $\beta 2m$ was mostly expressed in inclusion bodies. While it was expected that SUMO tag would increase the solubility of $\beta 2m$, it was not a surprise since several studies have shown that $\beta 2m$ is mainly expressed in inclusion bodies when using a bacterial expression system with *E. coli* (W. Chen, Gao, et al., 2010; Z. Chen et al., 2017; Garboczi et al., 1992). Despite this, $\beta 2m$ was produced as a fusion protein with the SUMO-His tag and then folded in arginine buffer which is a common method to refold proteins. Arginine buffer is widely used due to its ability to decrease non-specific hydrophobic interactions between partially folded or misfolded proteins intermediates (Tsumoto et al., 2004). This is critical since proper assembly and folding of the MHC-I/ $\beta 2m$ /peptide complex depends on regulated folding environment. After folding the $\beta 2m$ protein, an extra dialysis step in Bicine (pH 9.0) was performed to eliminate residual arginine from the protein mixture since it can interfere with downstream steps. During this process, the SUMO-His tag was cleaved with the ULP1 protease (Hickey et al., 2012). Following the removal of the

SUMO-His tag, soluble $\beta 2m$ was successfully separated from protein aggregates by size-exclusion chromatography resulting in a single ~ 11 kDa band confirmed by SDS-PAGE.

With both proteins purified, VHSV peptides from the nucleoprotein (Protein N) and glycoprotein (protein G) were chosen mixing different bioinformatic tools. The first tool used to select VHSV peptide candidates was NetChop 3.1, which is a proteasomal cleavage site prediction software specifically for MHC-I molecules when the 20S proteasome option is used, which was the case on this thesis. This is a neural network-based software that was trained to predict the probability of cleavage of MHC-I ligands at each amino acid position (Nielsen et al., 2005). The cleavage score ranges from 0 to 1, with values closest to 1 having higher confidence in the proteasomal cleavage prediction. In this chapter, scores equal or higher than 0.95 were selected to increase the chances to get a high-confidence prediction.

In addition to this prediction tool, NetCTL 1.2 was used to predict potential epitopes to be recognized by CD8⁺ T cells. This software has three parameters to predict these epitopes: proteasomal cleavage site, TAP transporter affinity and MHC-I binding affinity (Larsen et al., 2007). These parameters simulate the processing and presentation of antigenic peptides during the antigen presentation pathway from the production of peptides in the cytosol, ER transport and MHC-I loading. The peptides with highest scores generated by NetCTL 1.2 were selected, following the same reasoning mentioned previously, to ensure high-confidence prediction. From these predicted peptides, only the ones that had hydrophobic residues in position 2 and 9 were chosen, since it has been demonstrated that MHC-I anchors peptides with better affinity when they have these characteristics (Gfeller et al., 2023).

Finally, HADDOCK 2.4, a molecular docking software, was used to simulate the binding of the selected peptides to the rainbow trout MHC-I UBA and $\beta 2m$ complex. This software utilizes different parameters to determine the best model in which a high binding to MHC-I is preferred (Honorato et al., 2024) by analyzing the van der Waals energy, which determines the complementarity of VHSV peptides with MHC-I molecules in terms of steric fit, with negative scores indicating a better fit. Both protein N and G peptides had similar van der Waals energy, with peptide 1 for both proteins, being the ones that had lower energy (Protein N1: TMIGLFTQA and Protein G1: TVTFCGAEW). Another parameter analyzed was the electrostatic energy, where negative values indicate better charge-based interactions between VHSV peptides and MHC-I peptide binding groove (Zhou & Pang, 2018). As shown in Figure 2.6, peptide 3 for both protein N (NADNLMDIV) and G (LVQRNLVEI), had lower scores compared to the other peptides. In terms of buried surface area (BSA), which measures the stability of the binding between viral peptide and MHC-I, peptide 1 and 2 demonstrated higher values, for protein N (TMIGLFTQA and MIGLFTQAA, respectively) and peptide 2 and 3 for protein G (SIDLYSRKF and LVQRNLVEI). The reproducibility and consistency of binding was measured by the root mean square deviation (RMSD), with lower values having a better predicted fit (Bagaria et al., 2012). All peptides for both proteins had similar RMSD scores, proving the consistency of the results. Finally, the HADDOCK score was determined, which is the overall binding of VHSV peptides to MHC-I molecules, where negative scores indicate a stronger binding and better prediction of the peptides selected (Kastritis et al., 2014). As observed in Figure 2.6.1, the N protein peptide 1 (TMIGLFTQA) and 2 (MIGLFTQAA) had lower HADDOCK scores than peptide 3 (NADNLMDIV), indicating stronger binding from the first two candidates compared to peptide 3.

While, for protein G, peptide 1 (TVTFCGAEW) and 3 (LVQRNLVEI) had lower scores compared to peptide 2 (SIDLYSRKF) (Figure 2.6.2). Due to these results, peptides 1 and 2 were chosen for N protein and peptide 1 and 3 for G protein to be synthesized and used for *in vitro* refolding of MHC-I/ β 2m complexes.

As mentioned previously, peptide 1 – TMIGLFTQA and peptide 2 – MIGLFTQAA from N protein showed a more favorable docking prediction with the rainbow trout MHC-I UBA protein. While all protein N peptides have higher hydrophobicity, peptide 3 – NADNLMDIV, which was not chosen - has acidic and polar residues which may destabilize binding with MHC-I contributing to poor HADDOCK scores compared to peptide 1 and 2 (Garstka et al., 2015). In terms of G protein, peptide 1 - TVTFCGAEW and peptide 3 – LVQRNLVEI , both have hydrophobic residues, in comparison peptide 3 – SIDLYSRKF, that has hydrophobic residues in the anchor positions for MHC-I binding, but has charged and polar residues in other positions, which could result in less favorable binding to MHC-I (Calis et al., 2013). Thus, the biochemical composition of these peptides influenced the molecular docking results, supporting the selection of the specific VHSV peptides for further experiments (Guan et al., 2005).

After selecting the appropriate VHSV predicted peptides, the structure of rainbow trout MHC-I/ β 2m/VHSV-peptide complexes were modeled using AlphaFold2 via ColabFold and visualized in UCSF ChimeraX (Figure 2.7). The models showed binding of β 2m to the α 3 domain of the MHC-I heavy chain as demonstrated in resolved crystal structures of higher vertebrates and some fish species (Bjorkman P. J et al., 1987; W. Chen, Jia, et al., 2010; Sapert et al., 1991). In addition, the viral peptides were fitted within the peptide binding groove formed by the α 1 and α 2 domains of MHC-I molecules, supporting the published structures of this complex with other viral

peptides (Fremont et al., 1992; Zhang et al., 1992). This *in silico* structural prediction provide strong support for the peptide selection strategy.

To evaluate how temperature fluctuations may affect the stability of the MHC-I complexes in rainbow trout, molecular flexibility was calculated using CABSflex 2.0. This software simulates protein dynamics and flexibility estimating residue mobility by calculating root mean square fluctuations (RMSF) based on short Monte Carlo trajectories, which simulate protein movement (Kuriata et al., 2018). While temperature ranges can be tested, this is not a physical temperature like Celsius or Fahrenheit, instead, CABSflex 2.0 uses a temperature-like factor. In this chapter low (0.5), moderate (1.5) and high (2.5) values were used to mimic how temperature changes impact on the MHC-I/ β 2m/VHSV-peptide complex flexibility. As observed in Figure 2.8, RMSF values for MHC-I heavy chain showed minimal changes across residues at low temperatures (0.5), while at moderate temperatures, higher RMSF values were observed mostly in loop regions of MHC-I structure. This was likely due to the fact that these regions are more flexible than α -helices and β -sheets because they usually lack stabilizing hydrogen bonds and other interactions compared to secondary structures (Linse et al., 2020; Nagi & Regan, 1997). However, at high temperatures increased flexibility of MHC-I structure was observed in the α 3 domain, where β 2m binds. The α 3 domain of MHC-I is more flexible than the α 1 and α 2 domain because it has β -sheets and also has several loop regions increasing its flexibility which can led to less stability with temperature changes (Emberly et al., 2004). These flexibility in the α 3 domain suggests that the peptide binding groove of the MHC-I structure remains stable, while the binding to β 2m could be potentially disrupted at moderate to high temperatures.

In addition, at high temperatures (2.5), changes were also observed in the α -helices surrounding the peptide binding groove, causing differences between VHSV peptides complexed with MHC-I. For example, at this temperature, protein N – peptide 1 (TMIGLFTQA) showed high RSMF values in amino acid residues corresponding in both α -helices surrounding the peptide binding groove, suggesting poor binding and/or anchoring to this structure (Truong & Sgourakis, 2021). While this peptide contains hydrophobic residues that help with the anchoring to the peptide binding groove, it also has some bulky residues like methionine, phenylalanine and threonine that could result in suboptimal pocket fitting (Guan et al., 2005). In contrast, protein N – peptide 2 (MIGLFTQAA), produced flexibility in a small region of one α -helix surrounding the peptide binding groove compared to peptide 1. This could be because peptide 2 (MIGLFTQAA) lacks threonine and ends in a smaller alanine residue that could fit in the peptide binding groove of MHC-I better (Garstka et al., 2015; Guan et al., 2005). Among the G protein peptides, peptide 1 (TVTFCGAEW), maintained low flexibility compared to the other VHSV peptides, suggesting a better alignment with MHC-I peptide binding groove due to its hydrophobic and uncharged amino acid composition. On the other hand, peptide 3 (LVQRNLVEI) induced moderate flexibility in just part of the α -helices, likely due to the presence of charged amino acids which may generate electrostatic repulsion (Guan et al., 2005). These flexibility results can be correlated with the molecular docking data in which protein N – peptide 1 (TMIGLFTQA) and protein G – peptide 3 (LVQRNLVEI) showed slightly higher RMSD values and weaker electrostatic scores.

In contrast, β 2m showed similar patterns with all VHSV peptides bound to MHC-I, with higher fluctuations at high temperatures, mostly in loop regions (Figure 2.9). Finally, for the VHSV peptides alone, most remain stable at low temperatures, except for Protein G – peptide 3,

which had a higher flexibility between residue 6-9. At high temperatures, peptide flexibility increased in all peptides mainly in the C-terminal region (Figure 2.10). Biologically, the C-terminal region of antigenic peptides for MHC-I, especially at position 9, serves as a key anchoring residue in the MHC-I peptide binding groove, specifically in the F pocket (Achour, 2001; Pedersen et al., 2011). Peptides with suboptimal residues at or near position 9 such as polar or charged residues do not have the ideal hydrophobic anchoring, generating instability of the complex, in addition, secondary anchoring residues are also determinants for high-affinity binding the the MHC-I anchoring pockets (Sapert et al., 1991; Nguyen et al., 2021; Gfeller et al., 2023). These features, together with the effects of elevated temperatures, could explain the increased flexibility in this region. As temperature increases, there is an increase in molecular motion that can affect protein folding states destabilizing MHC-I complexes (Jappe et al., 2020; Lapidus, 2017).

Having identified promising VHSV peptide candidates through *in silico* analysis and evaluated their binding to MHC-I under different temperature conditions, the next step was to experimentally validate their ability to form stable complexes with purified MHC-I and β 2m recombinant proteins. Various refolding conditions were tested with the goal of optimizing complex formation to further assess how this complex unfolds at different temperatures. Finally, a refolding buffer containing L-arginine, reduced glutathione (GSH), oxidized glutathione (GSSH) and EDTA was chosen as optimal. This buffer is commonly used for the refolding of complexes having disulfide-bonded proteins with minimal aggregation, including MHC-I complexes (Ban et al., 2020; Shen et al., 2011). As observed in Figure 2.11, folded complexes of MHC-I/ β 2m/VHSV-predicted peptides were obtained with a molar ratio of 1:2:10, respectively after 48 h of incubation at 4C, with a better yield with protein G – peptide 1 (TVTF CGAEW). Although all four VHSV

predicted peptides by bioinformatics tools were able to form complexes with recombinant MHC-I and $\beta 2m$ under optimized conditions, the refolding efficiency was low. This is not surprising, since peptide-MHC refolding is often a challenge due to the need for correct peptide anchoring, binding affinity, appropriate folding kinetics; indeed, the assistance of chaperones is needed *in vivo* (Cresswell, 2000; Saikia et al., 2022). Among the VHSV peptides chosen, the highest yield was obtained with glycoprotein peptide 1 (TVTFCGAEW), which also showed favorable docking measurements and low conformational flexibility during molecular simulations (Figure 2.8 and Figure 2.10). While refolding was detectable, it is necessary to continue optimizing this *in vitro* assay to obtain better yields enough to continue with further experimentation.

To analyze the thermostability of the folded complexes and the ability of the VHSV peptides to increase the stability of the MHC-I complexes, differential scanning fluorometry (DSF) assays were performed. Unfortunately, all samples' unfolding profiles were similar to the refolding buffer control, indicating that the SYPRO Orange protein gel dye did not detect hydrophobic patches to bind. As mentioned previously, the refolding yield was low, which can explain the negative results by DSF, as the amount of refolded protein below the detection limit of the assay. Higher refolding yields will be needed to obtain these measurements.

In summary, this chapter explored the folding and stability of rainbow trout MHC-I/ $\beta 2m$ complex bound to VHSV-derived peptides and how it is affected by different temperatures. It was demonstrated the successful expression and purification of MHC-I and $\beta 2m$ proteins in a non-model organism like rainbow trout, representing a significant achievement since limited number of studies have characterized the folding and structure of these molecules in fish. In addition, using a combination of bioinformatic tools, VHSV peptide candidates were predicted to bind MHC-I

complexes, which was confirmed by AlphaFold2 analysis. Flexibility simulations using CABSflex 2.0 assessed the stability of the MHC-I complex under different temperatures, showing higher flexibility at elevated temperatures in key regions of the MHC-I molecules. Finally, *in vitro* refolding resulted in complex formation, confirming the results obtained with *in silico* approaches, although with low efficiency. This integrated *in silico* and *in vitro* approach supports future application in vaccine design and immunological research in aquaculture species.

Chapter 3: Influence of temperature effects on the antigen presentation protein dynamics in
Rainbow trout (*Oncorhynchus mykiss*) cell lines infected with VHSV IVb

3.1 Overview

Rainbow trout, face challenges due to climate-driven temperature fluctuations, increasing their susceptibility to pathogens like viruses. The major histocompatibility complex class I (MHC-I) pathway is essential for antiviral immunity by presenting intracellular antigens to cytotoxic lymphocytes to eliminate viral-infected cells. While previous studies show impaired gene expression of antigen presentation molecules at suboptimal temperatures, little is known about how temperature affects the protein function of antigen presentation molecules during viral infections. By integrating molecular and functional analysis, we aim to understand how temperature modulates the antigen presentation pathway in rainbow trout cell lines infected with viral hemorrhagic septicemia virus (VHSV) IVb. To accomplish this, rainbow trout cell lines were exposed to 4°C, 14°C, and 20°C and sampled at 1-, 4-, and 9-days post-infection (dpi). Then, the cell surface expression of MHC-I and its chaperone β 2-microglobulin (β 2m), the activity of the 20S proteasome subunit, β 2m release as part of the MHC-I/ β 2m recycling process and the secretion of IFN-I related to an antiviral response, were analyzed. Over the course of nine days, VHSV IVb-infected cells exposed to 14°C and 20°C, exhibited impaired MHC-I/ β 2m surface expression. In addition, proteasome activity was higher at 9 dpi at 14°C and 20°C, suggesting an increase in viral peptides processing. Release of β 2m was detected at 14°C and 20°C, which can correlate with the MHC-I recycling and trafficking. Also, differences in secretion of IFN-I were observed at 14°C and 20°C between control and infected cells, showing an antiviral response at these temperatures. However, no significant differences were observed among cells lines at 4°C, that could be the result of a reduced metabolic activity, impairing the virus ability to downregulate the antigen presentation pathway. These results highlight temperature-dependent modulation of antigen

presentation and viral processing, emphasizing the critical role of temperature in immune responses against viruses.

3.2 Introduction

As members of *Salmonidae* family, trout species are between the top freshwater teleost fish farmed in Canada, being Rainbow trout (*Oncorhynchus mykiss*) a popular species due to their fast-growing nature and economic value (D'Agaro et al., 2022). However, due to climate conditions a growing challenge to aquaculture is leading to losses of billions of dollars annually (Calvin et al., 2023). Heat waves and sudden temperature drops called cold snaps can cause physiological stress affecting fish health since they are poikilothermic species, which rely on their surrounding environment to both maintain and regulate internal biological functions such as the immune response (Liu et al., 2025; Michie et al., 2020; Mugwanya et al., 2022). Given that disease outbreaks are a persistent threat in aquaculture (Semple & Dixon, 2020), understand how suboptimal temperatures influence the immune responses of fish at a molecular level is crucial.

Adaptive immunity in mammals is a highly specialized response that allows the host to respond specifically to pathogens and generate memory immunity (Uribe et al., 2011). The endogenous antigen presentation pathway (EAPP) is one of the mechanisms in immunity used to present intracellular antigens such as viruses to T cells through the Class I Major Histocompatibility Complex (MHC-I) (Cunliffe & Trowsdale, 1987; Wearsch & Cresswell, 2013). All nucleated cells express MHC-I molecules and during the endogenous pathway, they present

intracellular antigens to CD8⁺ cells or cytotoxic T lymphocytes (CTL), triggering their activation and generating the eradication of infected cells in all tissues (Salter Russel D et al., 1989).

Antigen repertoire presented by MHC-I molecules is produced through the ubiquitin-proteasome system, or 26S proteasome, which is a large barrel-shaped protein mega complex composed of the 20S catalytic core and the 19S regulatory complex (Santambrogio et al., 2019). These peptides are then loaded onto the MHC-I molecules and together with its ER chaperone β 2-microglobulin (β 2m) are transported to the cell surface to present this peptides to CD8⁺ T cells (Blees et al., 2017b; Panter et al., 2012). The MHC/ β 2m molecules can be recycled in endocytic compartments and/or degraded into lysosomes. The recycling of the MHC molecules generates the reappearance of these proteins on the cell surface to continue their role as antigen presenting proteins (Montealegre & Van Endert, 2019b; Walseng et al., 2008). Intracellular pathogens such as viruses are capable of interfering with endogenous antigen presentation in numerous ways for example by inhibiting the proteasome ability to cleave peptides, blocking the transportation of peptides into the ER and/or disrupting the translocation of MHC-I complex to the cell surface (Yewdell & Hill, 2002).

In teleost fish the MHC genes are not complexed together in the same chromosome as other vertebrates (Dixon et al., 1995). Instead, there are two separate regions encoding for MH class I molecules in Rainbow trout called, *Onmy*-IA and *Onmy*-IB, where the classical class I gene UBA is expressed in the *Onmy*-IA locus among others (Shiina et al., 2005). The gene and protein expression of the MH molecules and the related chaperones have been observed in different immune and non-immune tissues as well as in Rainbow trout cell lines, both as basal levels and

after different stimuli (Dijkstra et al., 2003b; Kales et al., 2004; Kawano et al., 2010a; Sever et al., 2013, 2018b; Sever, Vo, Bols, et al., 2014).

For decades, the Viral hemorrhagic Septicaemia Virus (VHSV) has been known to cause acute systemic disease in aquatic species (Skall et al., 2005). The VHSV IVb strain was discovered in the North American great lakes in 2003 and is reported to have low pathogenicity in rainbow trout (Pham et al., 2013). The low mortality of VHSV IVb should make it a great candidate for research with rainbow trout cell lines, as it could generate an observable immune response without being overly cytopathic to the cell culture (Emmenegger et al., 2013).

Despite previous studies having demonstrated that suboptimal temperatures led to increased mortality of infected fish (Lopez et al., 2023; Vadboncoeur, Nelson, Hall, et al., 2023), there is limited research investigating how suboptimal temperatures modulate molecules of the antigen presentation pathway and how this could affect during an infection disease in fish. One study on rainbow trout monocyte/macrophage cell line (RTS11) concluded that total protein expression of MHC-I, $\beta 2m$, and secreted $\beta 2m$ significantly decreased in cells infected with IVa kept at 2°C (Sever, Vo, Lumsden, et al., 2014). In another study using a rainbow trout hypodermal fibroblast cell line (RTHDF) demonstrated delayed upregulation in *mhIa* and $\beta 2m$ gene expression in cells infected with VHSV IVb at 4°C (Abram et al., 2019). While these experiments provide valuable insights regarding the influence of suboptimal temperatures on the expression of the antigen presentation molecules, most of these studies have relied on PCR-based approaches to assess transcript levels of MHC-I and related molecules. As a result, there remains a significant knowledge gap regarding the molecular mechanisms and the functional dynamics of these proteins, and how they may be influenced by different temperatures following a viral infection. The

objective of this chapter was to investigate the cellular responses to infection with VHSV Ib under suboptimal temperatures. Specifically, we examined the effects of the temperature on the cell surface expression of MHC-I/ β 2m, the proteasome activity, and the secretion of β 2m and IFN-I. By integrating these functional and molecular analysis, we aim to provide a first approach into how temperature fluctuations modulate antigen presentation and protein trafficking in teleost fish. Understanding these mechanisms is crucial for improving disease management strategies in aquaculture, particularly in the context of climate change.

3.3 Materials and methods

3.3.1 Rainbow trout cell lines

The rainbow trout spleen monocyte-macrophages cell line (RTS11) (Ganassin & Bols, 1998), the gill epithelial cell line (RTGill-W1) (Bols et al., 1994) and the intestinal epithelial cell line (RTGut-GC) (Kawano et al., 2011) were cultured in 75 cm² or 175 cm² tissue culture flasks (Thermo Fisher). Cells were grown at 20°C in complete media consisting of Leibovitz's L-15 medium (L-15; Cytiva) supplemented with 10% (RTGill-W1 and RTGut-GC) or 20% (RTS11) fetal bovine serum (FBS; Corning) and 1% penicillin/streptomycin (Cytiva). Complete media was replaced every 7 days and cells were sub-cultured at 70-80% confluence. Passages were performed by adding 0.25% Trypsin-EDTA (Trypsin; Gibco) to collect adherent cells. Cells were then centrifuged at 300 *xg* for 5 minutes at 4°C, and the cell pellet was resuspended in complete media. Cells were counted in a Neubauer chamber with 0.4% Trypan Blue Stain (Gibco) for infection trials.

3.3.2 Viral propagation

Viral hemorrhagic septicemia virus (VHSV) IVb (CEFAS strain U13653) was propagated on confluent epithelioma papulosum cyprinid (EPC) cultures as described by (Lumsden et al., 2007). EPC cells were infected with diluted VHSV IVb in infection media comprised of L-15 medium supplemented with 2% FBS. Infection media was replaced with fresh complete media after 2 hours and media was collected after 10 days of infection. The media was filtered through a 0.22 µm filter and stored at -80°C for future use. The TCID₅₀/mL and pfu/mL were determined by the Poisson distribution.

3.3.3 Antibody production and purification

Polyclonal antibodies against rainbow trout MHC-I UBA (MHC-I), β2-microglobulin (β2m) and interferon type I (IFN-I) were produced in rabbit and chicken by Cedarlane Labs (Ontario, Canada) using synthetic peptides conjugated to Keyhole limpet hemocyanin (KLH). Three boosters with 0.2 mg of KLH-peptides were given at 21-d intervals. Serum was collected and titrated by ELISA using synthetic non-conjugated peptides. The polyclonal antiserums were affinity purified in a column containing protein A-agarose specific to non-conjugated synthetic peptides (5 mg).

3.3.4 Infection trial

Approximately 4×10^6 cells were seeded into 25 cm² tissue culture flasks with complete media and left to adhere at 20°C. Then, complete media was replaced with infection media (L-15 supplemented with 2% FBS) and cells were infected with VHSV IVb (MOI 1.0) as described by (Abram et al., 2019). Flasks were then incubated at 4°C, 14°C, or 20°C. Cells without VHSV IVb

were used as a control group for each respective temperature. After 1-, 4-, and 9-days post infection (dpi), cell media and adherent cells were collected by adding 0.25% Trypsin-EDTA. On each day of collection, a total of 0.5×10^6 cells/tube were used for flow cytometry analysis, and 0.2×10^5 cells were used for proteasome activity experiments. Cell media was stored at -80°C until use (Figure 3.1).

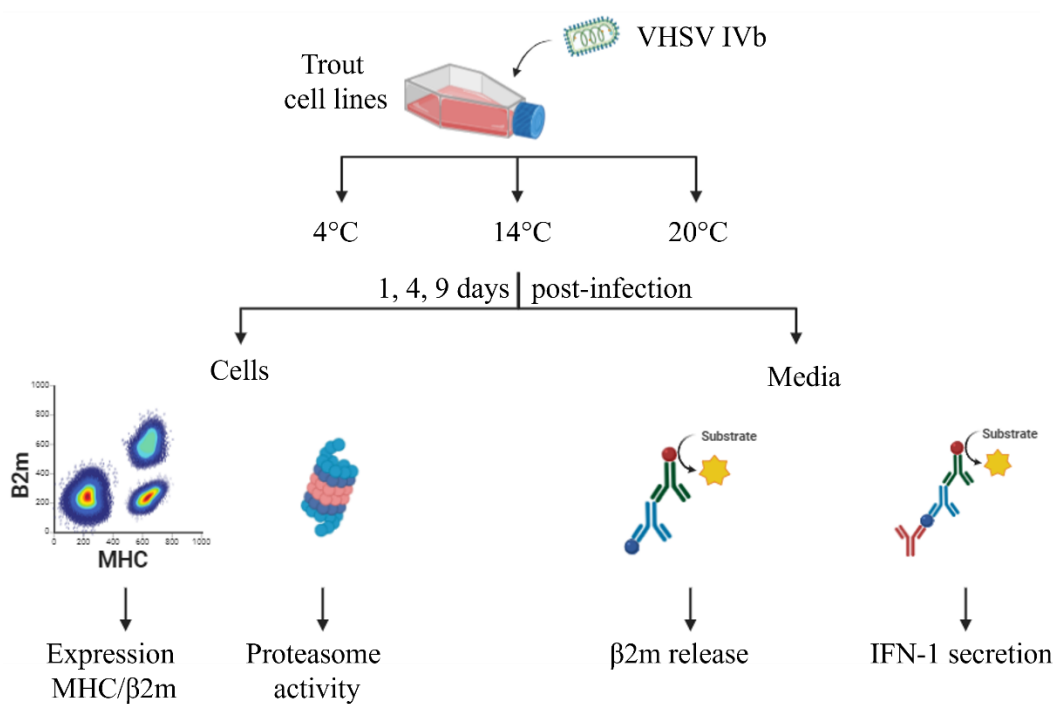


Figure 3.1. Schematic representation of infection trial. Trout cell lines RTGill-W1, RTS11 and RTGut-GC were infected with VHSV IVb and exposed to 4°C , 14°C and 20°C during 1, 4 and 9 days. After exposure, cells and media were collected to analyze different parameters of antigen processing and presentation. *Created with BioRender.com.*

3.3.5 Flow cytometry analysis

Non-specific binding sites were blocked by incubating 0.5×10^6 cells with FACS buffer (phosphate buffer saline; PBS supplemented with 2% FBS) for 10 minutes, followed by washing with ice-cold PBS and centrifugation at $300 \times g$ for 5 minutes at 4°C . Cells were then incubated with chicken anti-trout MHC-I antisera (35 ng/ μl), rabbit anti-trout $\beta 2\text{m}$ antisera (40 ng/ μl), or both polyclonal antibodies in FACS buffer for 1 hour at 4°C . Following incubation, cells were washed as previously described and incubated with donkey anti-chicken IgY conjugated to AlexaFluor®647 and/or donkey anti-rabbit IgG conjugated to AlexaFluor®488 at a 1:600 dilution in FACS buffer for 30 minutes at 4°C . After washing, cells were resuspended in FACS buffer and 1 $\mu\text{g/mL}$ of propidium iodide (PI) was added to evaluate cell viability. Unstained cells were used as negative controls, and cells stained with anti-chicken IgY (35 ng/ μl) and/or anti-rabbit IgG (40 ng/ μl) with corresponding secondary antibodies were used as isotype controls. Flow cytometry was performed using a BD FACSAria Fusion Flow cytometer (BD Biosciences) and the BD FACSDiva™ software. A total of 10,000 events from each sample was recorded.

3.3.6 Indirect Enzyme Linked Immunosorbent Assay (ELISA)

Ninety-six microtiter flat bottom plates (Immulon 2HB, Fisher Scientific) were incubated with 100 μl /well of cell media (from *section 3.3.4*) for 4 hours at room temperature. Plates were blocked with 300 μl /well of blocking solution (5% skim milk powder in Tris-buffered saline) and incubated for 1 hour at 37°C . Then, plates were incubated overnight at 4°C with 100 μl /well of rabbit anti-trout $\beta 2\text{m}$ antisera (1 $\mu\text{g/mL}$). The next day 100 μl of anti-rabbit IgY conjugated to biotin (BioRad #STAR121B) diluted to 1:1000 was added to each well, followed by 1 hour

incubation at room temperature in the dark. Afterwards, 100 μ L of streptavidin conjugated to horseradish peroxidase (HRP; Cedarlane #405210) diluted to 1:1000 was added to each well, followed by incubation for 1 hour at room temperature in the dark. Finally, plates were washed four times and incubated with 100 μ L/well of TMB Plus 2 (Cedarlane) for 30 minutes at room temperature in the dark, at which point 100 μ L/well of 0.2 M H_2SO_4 stop solution was added. Absorbance was measured at 450 nm on a Synergy H1 Hybrid Reader (BioTek Instruments). All samples were done in triplicates. Each antibody was diluted with 5% skim milk in TBS containing 1% Tween 20 (TBS-T; Sigma-Aldrich). Plates were washed four times with TBS-T between each incubation period unless stated otherwise. Blank wells on each plates containing infection media were used to determine net absorbance levels of extracellular β 2m. Serial dilutions of recombinant trout β 2m on each plate were used to plot a standard curve (Supplementary Figure S.1)

3.3.7 Western blot analysis

The secretion of β 2-microglobulin to the cell media was analyzed by western blot. Briefly, thirty microliters of cell media from control or infected cells exposed at suboptimal temperatures were loaded into a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Secreted proteins were then transferred to a nitrocellulose membrane using the Trans-Blot Turbo transfer system (Bio-Rad, USA), and complete transfer was confirmed by staining the blots with Ponceau-S (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid). Blots were incubated with 5% skim milk (w/v) in tris-buffered saline, pH 7.5, 0.1% Tween-20 (TBS-T), during 1 h at room temperature to avoid nonspecific binding. Then, blots were incubated with the polyclonal antibodies rabbit anti-trout β 2-microglobulin (3 μ g/ml), overnight at 4°C. Afterwards, goat anti rabbit IgG -

horseradish peroxidase conjugate secondary antibody (1:50.000, Sigma-Aldrich), was added to the blots during 1 h at room temperature protected from the light. The reaction was developed using the Pierce ECL Western Blotting Substrate kit (Bio-Rad, USA). Bands were visualized using the ChemiDoc XRS+ System (Bio-Rad, USA).

3.3.8 Proteasome activity

Proteasome activity was measured using the Proteasome Activity Assay Kit analyzing the cleavage of the fluorescent substrate LLVY-R110 (Sigma- Aldrich). Approximately 0.2×10^4 cells/well were resuspended in infection media, seeded into 96-well plates in duplicate, and left to adhere for 24 hours at room temperature in the dark. Infection media alone was used as background control. The next day, infection media was discarded and 100 μL /well of loading solution was added according to manufacturer's instructions, followed by 5 hours of incubation at room temperature in the absence of light. Two wells of loading solution served as blanks to establish background fluorescence. The mean fluorescence intensity (MFI) was measured on a Synergy H1 Hybrid Reader (BioTek Instruments) at excitation and emission wavelengths of 490 nm and 525 nm, respectively.

3.3.9 Sandwich Enzyme Linked Immunosorbent Assay (ELISA)

Ninety-six microtiter flat bottom plates (Immulon 4HBX, Fisher Scientific) were coated with 100 μL /well of rabbit anti-trout IFN-I capture antibody (Cedarlane #200527) at a 1:500 dilution in coating buffer (sodium carbonate, sodium bicarbonate, pH 9.6) and incubated for 4 hours at room temperature. Plates were then blocked with 300 μL /well of blocking solution and incubated for 1 hour at 37°C. Then, wells were incubated at 4°C overnight with 100 μL of cell

media (from *section 3.3.4*) or recombinant trout IFN-I serially diluted in infection media with concentrations ranging from 800 pg/mL to 6.25 pg/mL (Abram et al., 2019). The next day, 100 μ L of chicken anti-trout IFN-I detection antibody (Cedarlane #200723) at a 1:1000 dilution was added to each well and plates were incubated for 3 hours at room temperature. Then, plates were incubated with 100 μ L/well of anti-chicken IgG conjugated to biotin (Cedarlane #YI4054368E) diluted to 1:2000, followed by incubation with 100 μ L/well of streptavidin conjugated HRP (Cedarlane #405210) diluted to 1:1000 for 1 hour at room temperature in the dark. Plates were then washed four times and incubated with 100 μ L/well of TMB Plus 2 (Cedarlane) at room temperature in the dark for 30 minutes, at which point 100 μ L/well of stop solution was added. Absorbance was measured at 450 nm on a Synergy H1 Hybrid Reader (BioTek Instruments). All samples were done in triplicates, and all antibodies were diluted with 5% skim milk in TBS-T. Plates were washed four times with TBS-T between each incubation period unless stated otherwise. Serial dilutions of recombinant trout IFN-I on each plate were used to plot a standard curve (Supplementary Figure S.2), and interpolation of the linear trend line equation was used to calculate the concentration of IFN-I in each sample.

3.3.10 Statistical analysis

Collected data from each assay was graphed and statically analyzed with the GraphPad Prism 10 software. Significant differences between temperature and treatment at each time point were determined using 2-way ANOVA with Tukey's multiple comparison test.

3.4 Results

3.4.1 Cell surface expression of MHC-I/ β 2m under suboptimal temperatures after infection with VHSV IVb

To elucidate the implication of suboptimal temperatures in the dynamics of the antigen presentation pathway during an infection in rainbow trout, RTGill-W1, RTS11 and RTGut-GC cells were exposed to 4°C, 14°C and 20°C and then infected with the viral hemorrhagic septicemia virus (VHSV) IVb for 1, 4 and 9 days. After each time point, cell surface expression of MHC-I/ β 2m was analyzed by flow cytometry, resulting in three distinct populations: 1) double-positive cells expressing both markers (MHC-I/ β 2m), single-positive cells expressing only MHC-I, and 3) double-negative cells lacking both molecules (Figure 3.2). Over the course of nine days, VHSV IVb-infected cells exposed to 14°C showed a decrease of MHC-I/ β 2m surface expression, as indicated by an increase in double-negative cell population across all cell lines, compared to control groups, and other temperature conditions. This effect was more pronounced in RTS11 cells (45%), followed by RTGill-W1 (40%), and RTGut-GC (5%) (Figure 3.3). Concurrently, a progressive decrease in the double-positive cell population was observed over time at 14°C in RTS11 and RTGill-W1 cells following VHSV IVb infection, but no differences were shown in RTGut-GC cell line. Notably, at 20°C, a slight increase in double-negative cells was observed in RTGill-W1 cells at 4 days post-infection (Figure 3.3A). Conversely, in RTS11, the double-positive population was higher at 4°C compared to 14°C after infection (Figure 3.3B). No other significant differences were observed among cells lines at 4°C. In addition to analyzing MHC-I/ β 2m cell surface expression, cell viability was evaluated, showing a decrease starting at 1- or 4 days post-

infection with VHSV IVb in RTGut-GC and RTGill-W1 cells, respectively, but not in RTS11 at 14°C (Figure 3.4).

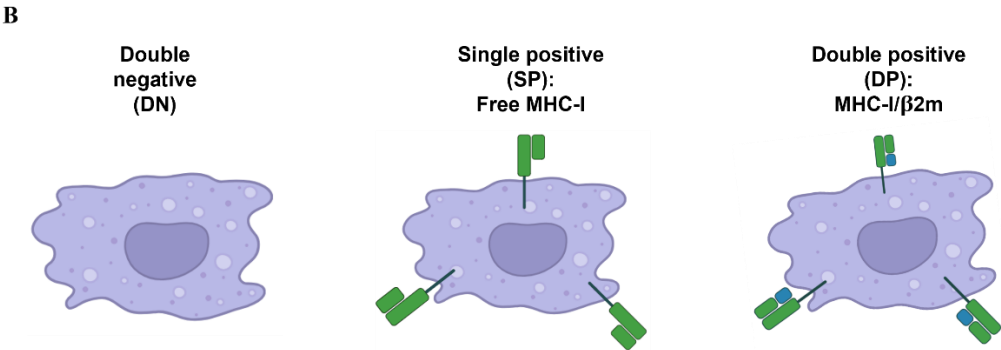
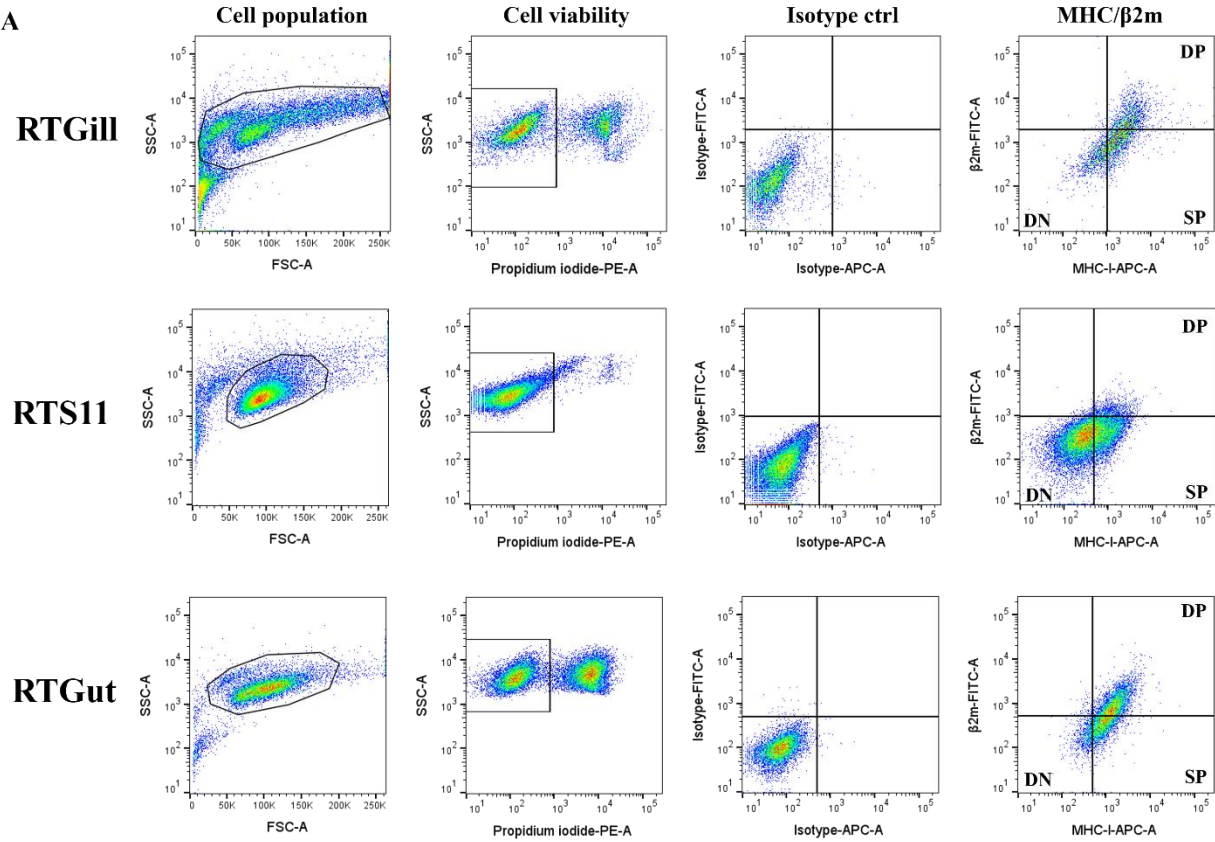
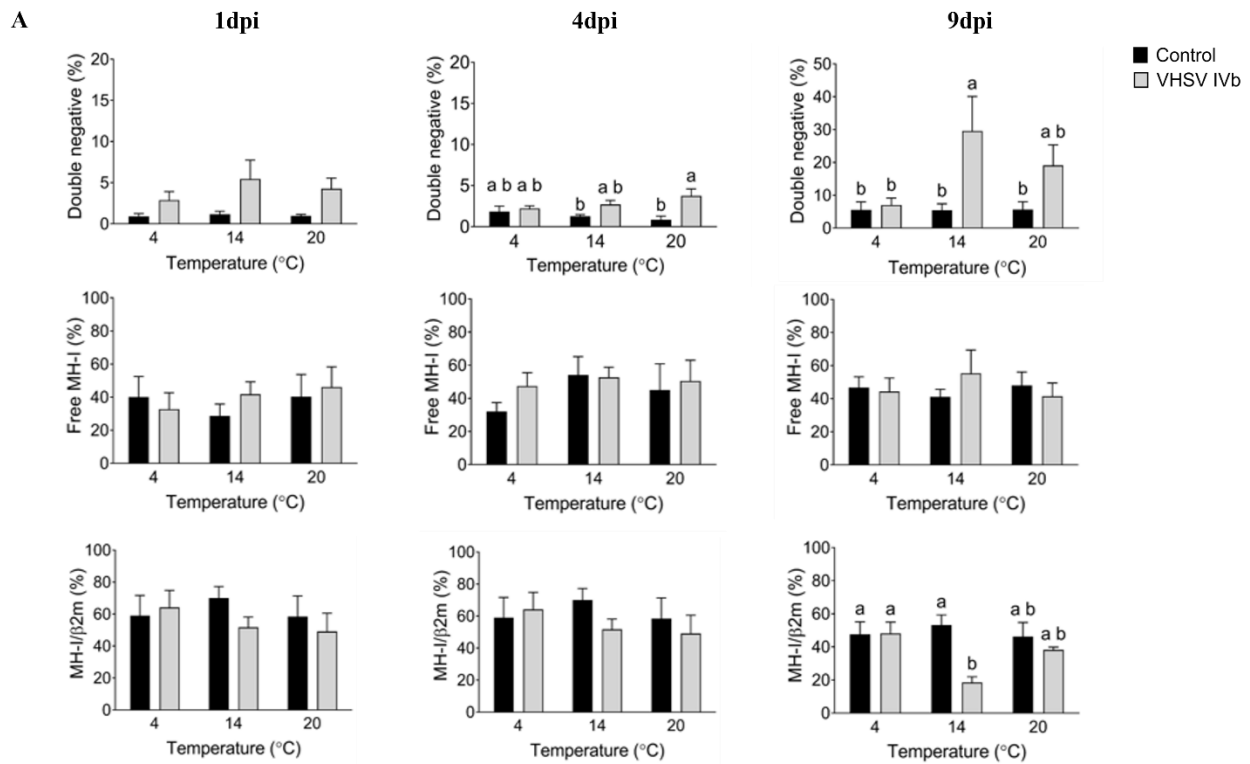
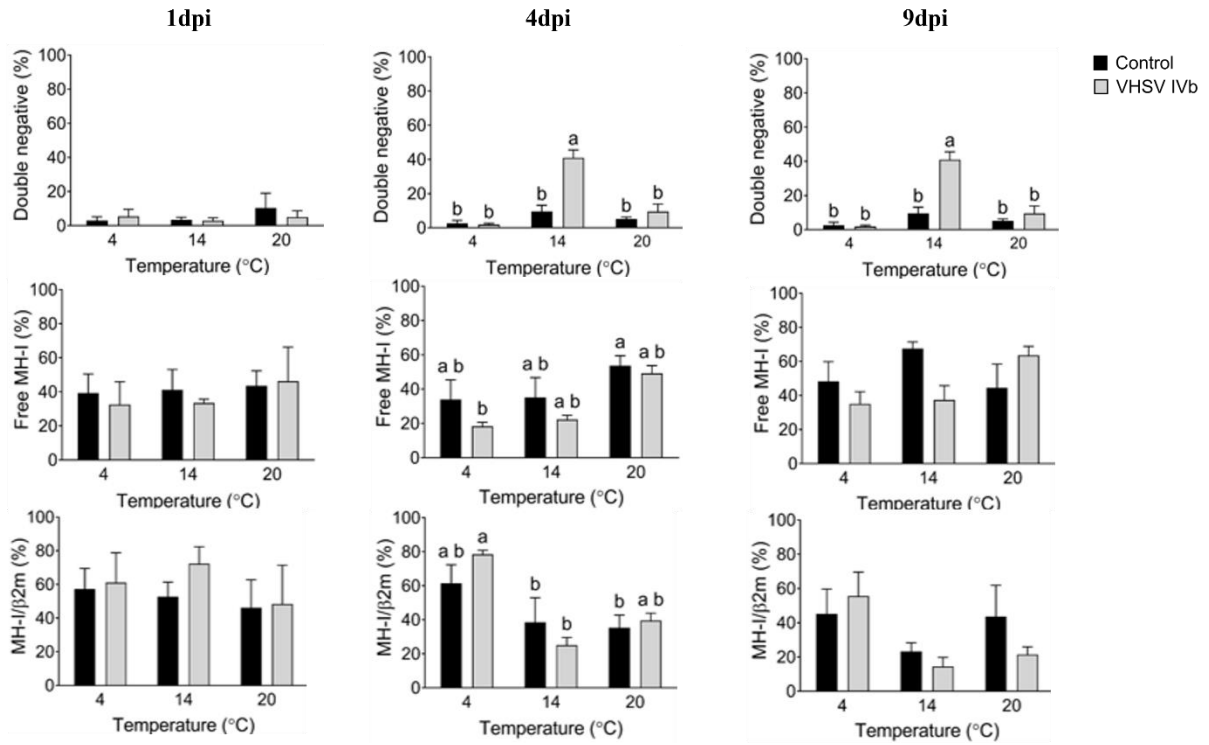


Figure 3.2. Flow cytometry gating procedure. (A) Cell surface expression of MHC-I/β2m complexes was analyzed in rainbow trout cell lines by flow cytometry. Cell populations were gated by size (FSC-A) and complexity (SSC-A) and the cell viability was evaluated using propidium iodide. From the live cell population (propidium iodide negative), MHC-I/β2m expression were analyzed. (B) Different populations are shown in quadrants: double negative (DN), double positive (DP) and single positive (SP). Isotype control for each antibody was used.



B



C

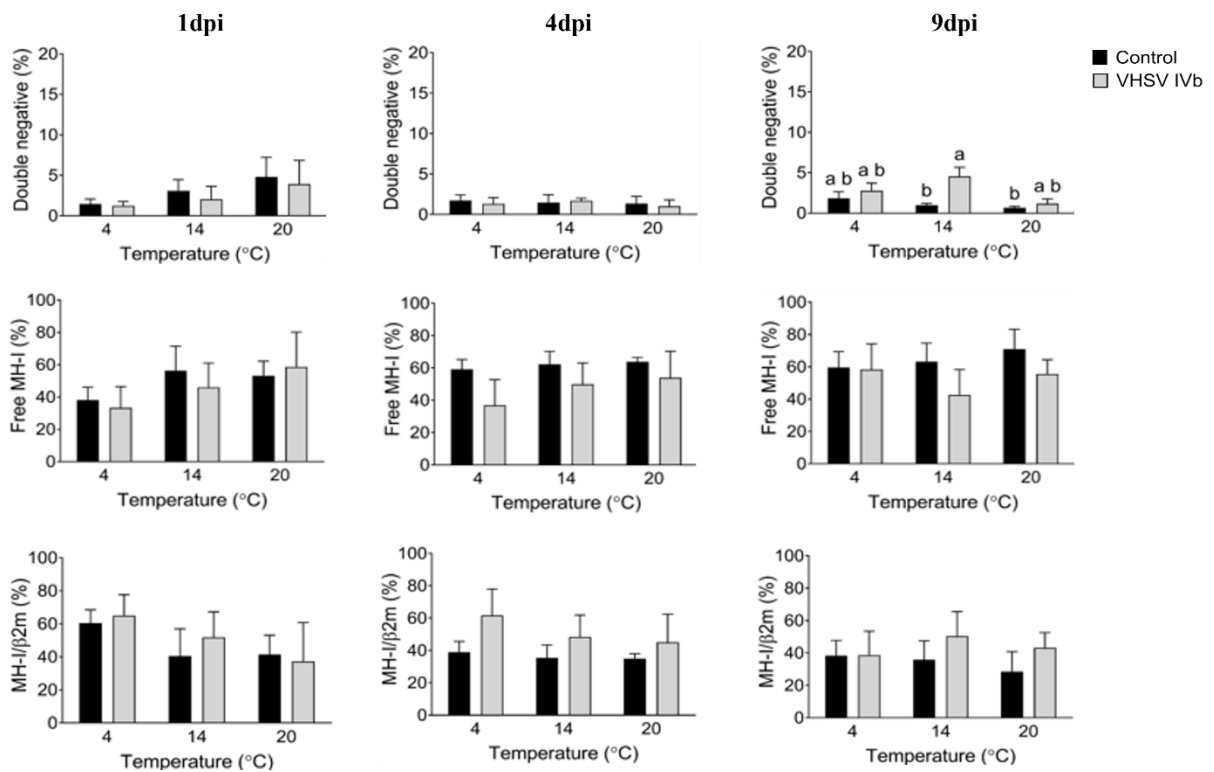


Figure 3.3 Cell surface expression of MHC-I/β2m. Rainbow trout cell lines RTGill-W1 (A), RTS11 (B) and RTGut-GC (C) were exposed to suboptimal temperatures during an infection with VHSV IV to then analyze the cells surface expression of MHC-I/β2m by flow cytometry after 1-, 4- or 9-days post-infection (dpi). Different letters indicate statistically significant differences ($p < 0.05$; two-way Anova, $N = 4$).

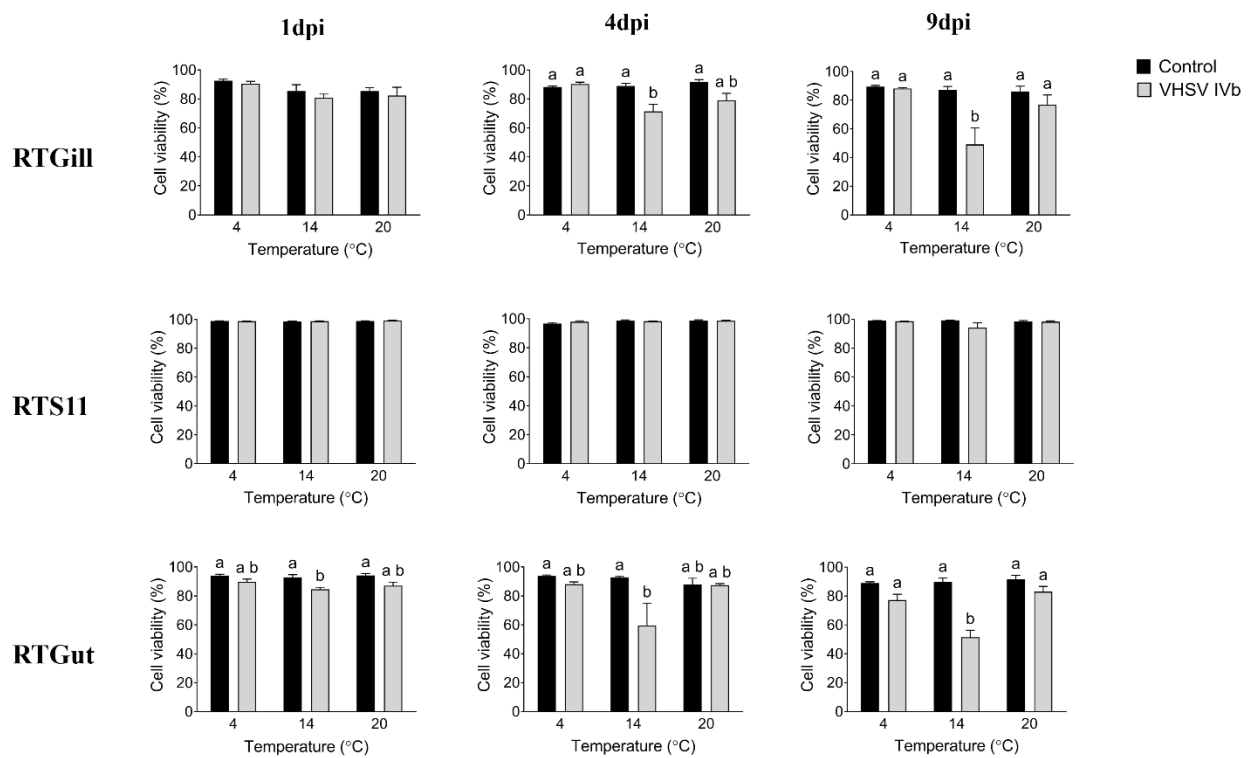


Figure 3.4. Rainbow trout cell lines viability. Propidium iodide was used to analyze the cell viability of RTGill-W1, RTS11 and RTGut-GC after exposure to suboptimal temperatures during

an infection with VHSV IVb for 9 days. Different letters indicates statistical differences between groups ($p < 0.05$; two-way Anova, $N = 4$).

3.4.2 Release of β 2-microglobulin in response to VHSV IVb infection at suboptimal temperatures

During the trafficking of the MHC-I/ β 2m complex from the cell surface, a proportion of β 2-microglobulin is released into the extracellular environment. An indirect ELISA was performed to evaluate if VHSV IVb interferes with the MHC-I/ β 2m recycling after the exposure to different temperatures. As shown in Figure 3.5, β 2m levels in the cell culture media increased starting at 4 days post-infection in RTGill-W1 cells at 14°C compared to both the control group and cells exposed to 4°C and 20°C. The amount of β 2m released into the extracellular environment increased from 30 ng/ml up to 400 ng/ml by 9 days post-infection at 14°C (Figure 3.5). In contrast, no significant differences were observed in RTS11 cells when comparing β 2m levels between control and infected groups. However, a significant difference was detected between infected RTS11 cells at 4°C and 14°C, beginning at 1-day post-infection, with β 2m levels reaching 300 ng/ml by day 9 post-infection (Figure 3.5). No significant differences were observed during the release of β 2m in RTGut-GC cell line (Figure 3.5). Western blot analysis of β 2m release from rainbow trout cell lines showed one pure band at the expected molecular weight for this protein (Figure 3.6).

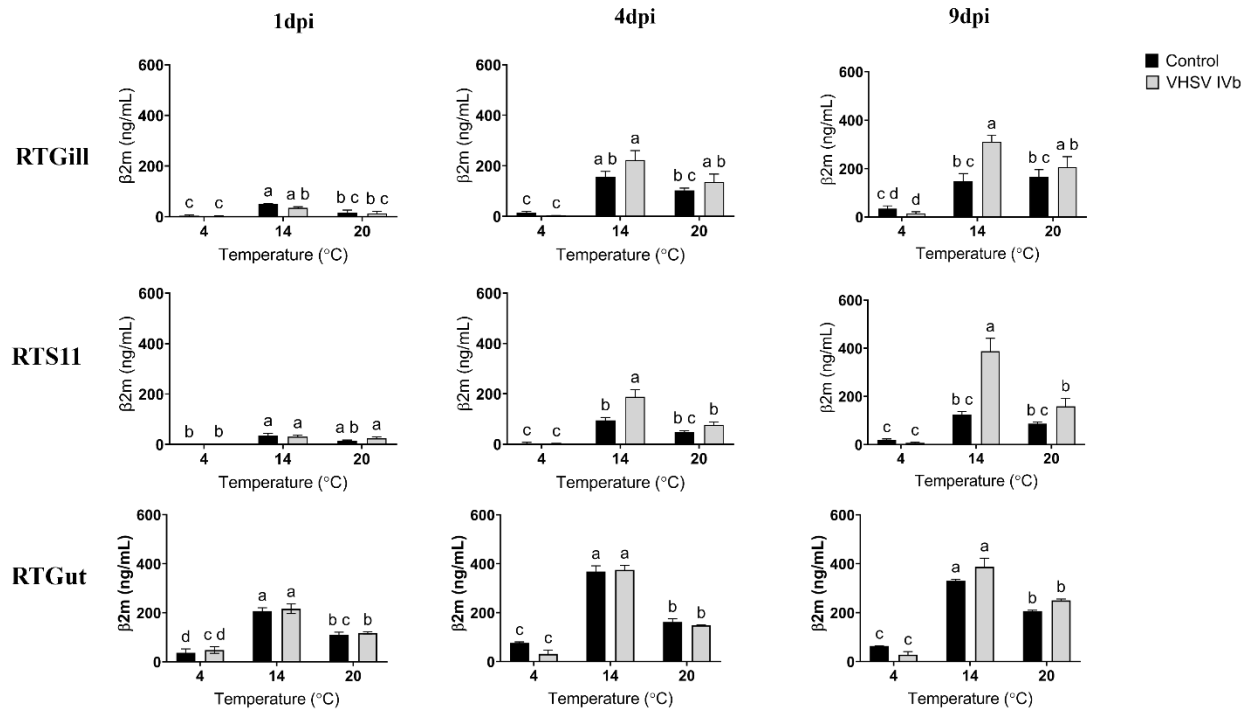


Figure 3.5. $\beta 2$ -microglobulin release to the extracellular space. Indirect ELISA was performed to analyze the release of $\beta 2m$ to the cell media from rainbow trout cell lines exposed to suboptimal temperatures under an infection with VHSV IVb. $\beta 2m$ release was quantified using a standard curve prepared with recombinant $\beta 2m$ (section 2.3.3). Statistically significant differences between groups are shown with different letters. A two-way Anova analysis was used to determine significant differences (p < 0.05; N = 4).

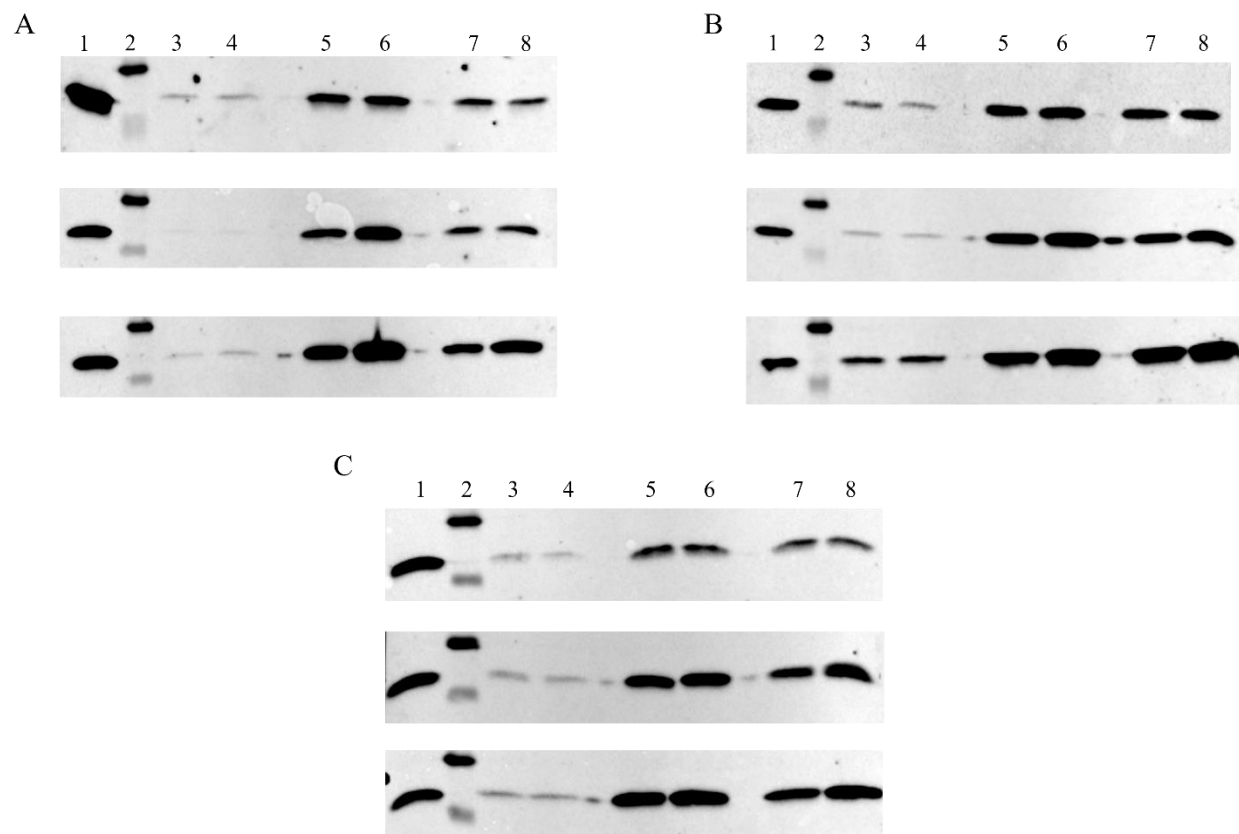


Figure 3.6. Western blot analysis of β 2-microglobulin release. The specificity of the anti- trout β 2m antibody was evaluated by western blot in cell media from RTGill-W1 (A), RTS11 (B) and RTGut-GC (C) exposed to suboptimal temperatures under an infection with VHSV IVb. Recombinant β 2m was used as positive control (lane 1). A molecular weight marker was used (lane 2), and samples were loaded in the following order: control groups at 4C (lane 3), 14C (lane 5) and 20C (lane 7); infected groups at 4°C (lane 4), 14°C (lane 6) and 20°C (lane 8).

3.4.3 Proteasome activity in VHSV IVb-infected rainbow trout cells under suboptimal temperatures conditions

To assess whether VHSV IVb infection disrupts the proteasome system and how suboptimal temperatures influence its function, the activity of the 20S proteasome subunit was analyzed. Results showed that after 9 days of infection at 14°C, there is a significant up-regulation of the proteasome activity in RTGill-W1 and RTS11 cells compared to the non-infected group, however, no differences were observed at other temperatures nor time points (Figure 3.7). In addition, significant differences were observed in RTGill cells at 4 dpi when cells were exposed to 14°C compared to 4°C, similarly this was observed at 9 dpi between cells incubated at 20°C compared to lower temperatures (Figure 3.7). No differences were observed in RTGut-GC cells (Figure 3.7).

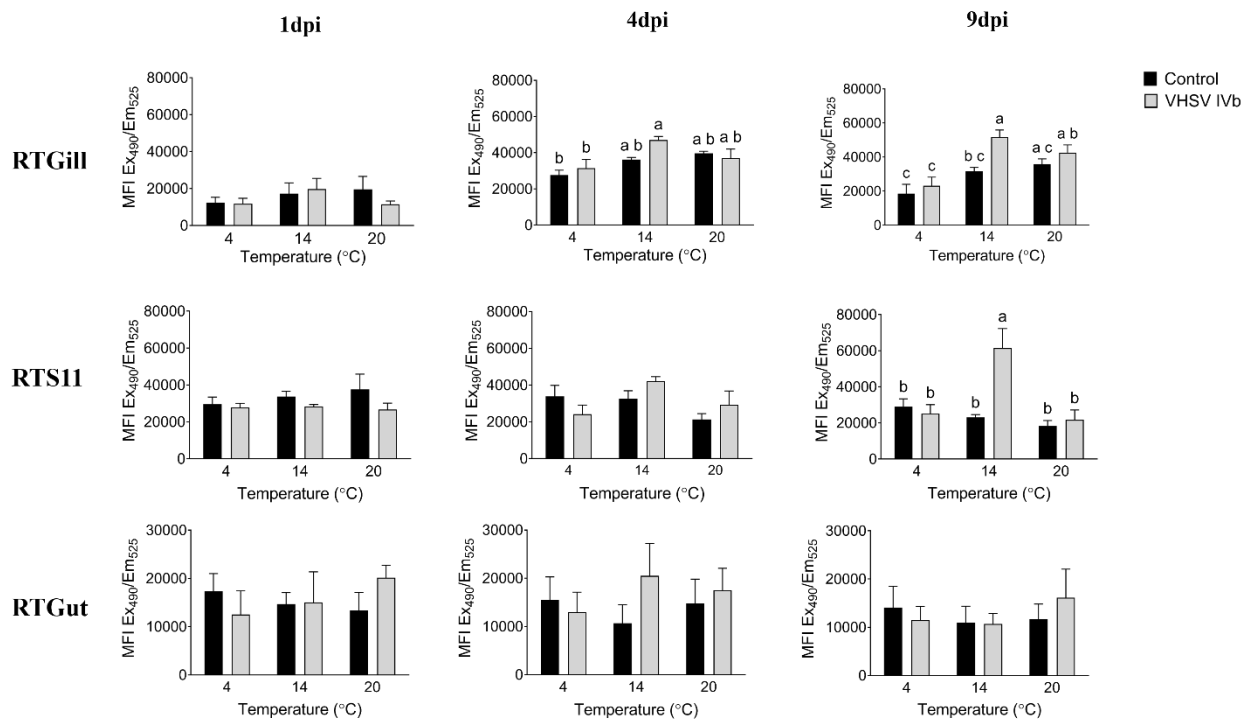


Figure 3.7. Proteasome activity analysis. Rainbow trout cell lines were exposed to suboptimal temperatures during an infection with VHSV IVb for 9 days. After each time point, the 20S proteasome activity was evaluated. Significant differences between groups are shown with different letters using a two-way Anova ($p < 0.05$; $N = 4$).

3.4.4 Antiviral response under suboptimal temperatures by the secretion of type I interferon

Viral infections elicit a range of immune responses, including the induction of antiviral cytokines such as type I interferon (IFN-I), which induces the endogenous antigen presentation pathway. To evaluate the role of IFN-I during VHSV IVb infection under suboptimal temperature conditions, secretion was measured using a sandwich ELISA (Figure 3.8). No detectable IFN-I expression was observed at 1 day post infection (dpi) in RTGill-W1 and RTS11 cell lines, and IFN-I levels in RTGut-GC showed no differences across treatment groups. However, from 4 dpi to 9 dpi, significantly elevated levels of IFN-I were detected in RTGill-W1 (Figure 3.8) and RTS11 (Figure 3.8) at 14°C and 20°C, compared to the control. In RTGut-GC, a significant increase in IFN-I was observed only at 14°C on day 4 post-infection, with no differences detected at 9 dpi (Figure 3.8).

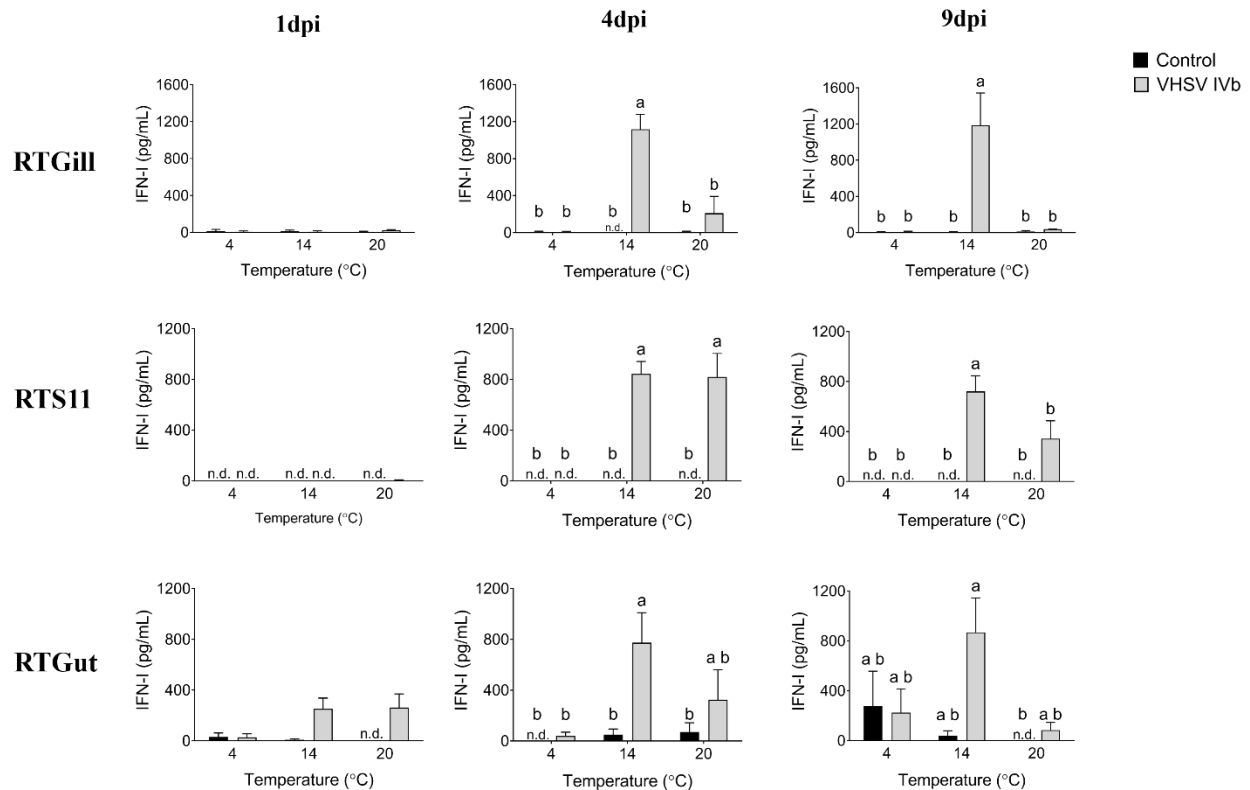


Figure 3.8. IFN-I secretion. After the exposure to suboptimal temperatures during an infection with VHSV IVb, the secretion of IFN-I from rainbow trout cell lines was evaluated by sandwich ELISA. Secretion of IFN-I was quantified using standard curve with recombinant IFN-I. Significant differences were calculated using a two-way Anova ($p < 0.05$; $N = 4$).

3.5 Discussion

Understanding the impact of suboptimal temperatures on antigen presentation during viral infections is crucial not only to gain knowledge into fish immunology but also to elucidate the overall effect on fish health, especially nowadays where temperature fluctuations are becoming more common due to climate change. In this chapter, how suboptimal temperatures affect different

steps of the MHC-I antigen processing and presentation pathway in VHSV-infected rainbow trout cell lines was investigated. In this chapter, the cell surface expression of MHC-I/ β 2m, the release of β 2m to the extracellular space, the proteasome activity and IFN-I secretion were also examined, giving overall insight of the immunological disruptions triggered by VHSV IVb infection under suboptimal temperatures.

The most interesting and consistent result across the three rainbow trout cells lines – RTGill-W1, RTS11, and RTGut-GC - was the decrease of the cell surface expression of MHC-I/ β 2m complexes after exposure to 14°C following VHSV IVb infection (Figure 3.3). This suppression observed with the increase of the double-negative population by flow cytometry (lacking both MHC-I and β 2m expression) was also associated with the decrease in the double positive-population. The observed results were more pronounced in RTS11, a monocyte/macrophage cell line from the spleen (Ganassin & Bols, 1998), and RTGill-W1, which originates from gill epithelial cells (Bols et al., 1994), compared to the gut epithelial cell line, RTGut-GC (Kawano et al., 2011). The latter showed a more stable expression of MHC-I/ β 2m across all temperatures, suggesting that there is a possible cell-line specific sensitivity to thermal stress during an infection with VHSV IVb (Konjar et al., 2017; Segrist & Cherry, 2020).

As mentioned previously, RTS11 is a monocyte/macrophage cell line from rainbow trout spleen (Ganassin & Bols, 1998) and thus has antigen-presenting capabilities that are intrinsically dependent on functional MHC-I pathway to initiate cytotoxic T cell responses (Brubacher et al., 2000; Kawano et al., 2010b). The higher suppression of MHC-I/ β 2m on the cell surface on this cell line suggests that antigen-presenting cells (APCs) may be vulnerable to temperature-mediated stress during infections (Evans et al., 2015; Abram et al., 2019; Doppegieter et al., 2024; Maloney

& Duffy, 2024). Similar research has shown that MHC-I was reduced at transcript levels in other under cold stress (Rodrigues et al., 1998; Kales et al., 2006). In this chapter, the fact that moderate suboptimal temperature (14°C) led to the decrease of MHC-I/β2m cell surface expression suggests the thermal range in which efficient antigen presentation occurs in the rainbow trout cell lines tested. As shown in Figure 3.3, infected cells at 14°C had higher suppression of MHC-I/β2m molecules on the cell surface. This suppression could be due to failure at different steps during the antigen presentation pathway, which is a common strategy used by viruses to evade the immune system (Yewdell & Bennink, 1999; Yewdell & Hill, 2002). One possibility is that moderate suboptimal temperatures (14°C) together with an infection with VHSV IVb causes impairment of the proper folding and assembly of MHC-I heavy chain with β2m and viral peptides, a process that is also dependent on other ER chaperones such as tapasin (Boeren et al., 2023; Wu et al., 2025). If there is any loss of function of these molecules, the folding of MHC-I complexes would be compromised, leading to an unstable or low-affinity peptide-MHC-I complexes which are more probable to be retained intracellularly, rapidly internalized and or degraded (Montealegre et al., 2019a; Zagorac et al., 2012). The ability of VHSV IVb to interfere with the expression of MHC-I/β2m at 14°C compared to other temperatures, despite high proteasome activity and interferon signaling (as discussed later in this section), supports the notion that the interference may be at the level of folding, stabilization, and/or trafficking of the MHC-I complexes and not upstream peptide generation (Hewitt, 2003; Peters & Sperber, 2009). Interestingly, at 4°C, which is well below optimal for these cell lines, the reduction of MHC-I/β2m complexes on the cell surface of RTS11 was not seen compared to 14°C, both in control and infected groups. This may suggest a metabolic “slow-down” that affects both viral replication and cellular pathways, creating an equilibrium in

which neither the virus nor the cellular immune responses are active enough to observe antigen presentation impairment (Abram et al., 2017; Appenheimer & Evans, 2018). On the other hand, at 20°C, which is the optimal temperature for rainbow trout cell lines such as RTS11, the cell surface expression of MHC-I/β2m was relatively stable during the 9 days of infection with VHSV IVb suggesting that higher metabolic rates may favor more efficient cellular immune responses, including antigen processing and presentation, at a temperature that causes slower or not that efficient viral replication (Jang et al., 2025; Kang et al., 2024).

Similarly, the rainbow trout cell line RTGill-W1 showed a significant decrease in MHC-I/β2m surface expression at moderate suboptimal temperature exposure (14°C) 9 days post-infection with VHSV IVb (Figure 3.3A). Studies have evaluated the role of gill epithelial cells demonstrating the important role in engulfing pathogen, expressing antigen presentation markers, and implication on cytotoxic immune responses due to the presence of CD8⁺ T cells in gills epithelium (Haugarvoll et al., 2008; Kato et al., 2018; Slattery et al., 2023). Compared to 4°C and 20°C, the expression of these molecules was stable as seen in RTS11, supporting the idea that both cellular metabolic rates and optimal temperatures for viral replication are crucial during immune activation. RTGut-GC cells, on the other hand, did not have large effects on the cell surface expression impairment of MHC-I/β2m (5%) compared to RTS11 (45%) and RTGill-W1 (40%) at 14°C at 9 dpi (Figure 3.3C). Similarly, neither 4°C nor 20°C showed significant differences among groups. This result suggest that gut epithelial cells have a distinct regulatory pathway to respond to viral infections that is not as dependent on the antigen presentation pathway or that their ability to present antigenic peptides is not that strong compared to the antigen-presenting cell line RTS11 or the epithelial cells from the gills (Porter et al., 2022).

The suppressed surface expression of MHC-I/ β 2m can be correlated with the observed increase in extracellular β 2-microglobulin at 14°C, particularly in RTGill-W1 and RTS11, in the infected groups (Figure 3.5). β 2-microglobulin dissociation from MHC-I complexes has been associated with several processes like MHC-I recycling or destabilization of the complex being both are sensitive to temperature and infection (Montealegre et al., 2015b). The depletion of MHC-I/ β 2m molecules and increase of β 2m secretion levels observed in this chapter suggests that the MHC-I/ β 2m complexes may be destabilized under VHSV IVb infection at a moderate suboptimal temperature. A possible evasion mechanism of VHSV to destabilize these complexes could be the interference in TAP and/or tapasin function leading to an impairment of loading of high-affinity peptides resulting in less stable MHC-I complexes (Fruh et al., 1995; Park et al., 2004). This would increase the internalization/recycling rate of MHC-I molecules within the cells, and together with potential increase in protein degradation, will result in less MHC-I molecules on the cell surface and more β 2m release in the extracellular space (Montealegre et al., 2015, 2019a; Montealegre & Van Endert, 2019b). Therefore, the possible evasion mechanism of VHSV at temperatures in which the viral replication is optimal while the immune response is impaired creates the perfect scenario to disrupt the antigen presentation pathway in RTS11 and RTGill-W1 (Abram et al., 2017; Jang et al., 2025; Kang et al., 2024). On the other hand, no differences were observed in RTGut-GC cells, which also showed minimal changes in the MHC-I/ β 2m surface expression, suggesting tissue-specific regulation of the antigen processing and presentation, and perhaps different roles in antiviral immune responses in rainbow trout (Konjar et al., 2017).

Different studies have observed that the release of β 2m is inhibited at lower temperatures (Dirscherl et al., 2022; Kales et al., 2006; Ljunggren et al., 1990), supporting the results seen here

in which at 4°C, minimal β 2m release was observed across cell lines and no differences were observed between groups. On the other hand, at 20°C, both control and infected groups had an increase of β 2m levels compared to 4°C but not to 14°C, which could be due to the normal recycling of cell surface receptors like MHC-I molecules (Dirscherl et al., 2022). As mentioned previously, 20°C corresponds to the optimal temperature for cell growth and thus normal metabolism of all the tested rainbow trout cell lines, giving a more favorable immune response against VHSV where protein folding, peptide loading and trafficking are less disrupted (Baas et al., 1992). Moreover, 20°C is considered a suboptimal temperature for VHSV replication that could end in a reduced viral activity resulting in less viral interference of the antigen presentation pathway (Jang et al., 2025). Additionally, higher temperatures can induce the expression of heat shock proteins (HSPs) that are known to assist in the protein folding and the prevention of protein aggregation. These proteins could be providing a better folding environment at 20°C compared to lower temperatures such as 4°C and 14°C (Murshid et al., 2012).

Interestingly, proteasome activity, which is essential during the generation of antigenic peptides to be loaded onto MHC-I molecules (Sijts & Kloetzel, 2011), was only upregulated at 14°C in the infected groups in RTGill-W1 and RTS11 at 9 days post-infection with VHSV IVb but it was not observed at 4°C or 20°C, nor in the RTGut-GC cell line (Figure 3.7). This could be due to increase in peptide generation provoked by viral stress during the exposure to moderate suboptimal temperatures (McCarthy & Weinberg, 2015). However, this upregulation in the proteasome activity occurred together with the impairment of MHC-I/ β 2m expression, suggesting the possibility that while peptides are being produced, they are not being loaded effectively onto MHC-I (Domnick et al., 2022) or peptides with low affinity were being generated (Henderson et

al., 1992). This discrepancy could be explained by the idea that under moderate thermal stress, upstream steps of the antigen presentation pathway are not disrupted but downstream steps like peptide loading and/or peptide presentation are impaired. While the results show this upregulation at a moderate suboptimal temperature (14°C), other studies have observed that there is an increase in proteasome activity at colder acclimation temperatures. In fact, in spotted wolffish, the 20S proteasome activity was higher at 4°C compared to 8°C and 12°C (Lamarre et al., 2009). Similarly, transcriptomic analysis in killifish showed an upregulation of the ubiquitin-proteasome system during cold acclimation, suggesting an increase in protein degradation under cold stress (Podrabsky & Somero, 2004). However, the findings here showed that only under moderate temperatures exposure and during a viral infection, the proteasome activity is upregulated, while at 4°C - the coldest condition tested – showed no differences between groups. This could suggest that extreme cold impairs cellular metabolism or that the temperature for proteasome activation is cell-type and context dependent, particularly during viral infection (Lamarre et al., 2009; Nombela et al., 2019; Podrabsky & Somero, 2004). It is important to note that cell lines were used in this study, not whole tissues and also they were not acclimated for long periods but exposed to infection under thermal stress, which could limit the development of thermal compensation observed in long-term cold-acclimated fish (Lamarre et al., 2009). It is also important to mention that the 20S proteasome activity kit used in this thesis detects only chymotrypsin-like activity of the constitutive 20S proteasome. In mammals, induction with cytokines such as IFN- γ and type I interferons, generates the formation of the immunoproteasome which is in charge of producing antigenic peptides supposedly with better affinity for MHC-I molecules (Ferrington & Gregerson, 2012). Although some studies have shown that while proteasome activity is required for antigen

presentation, immunoproteasome is not essential (Morel et al., 2000; Van den Eynde & Morel, 2001). However, it is not fully known if the protein degradation pathway is exactly the same in teleost fish, therefore it is necessary to have more specific assays to distinguish between constitutive and activated proteasome activity in teleost fish.

Finally, we analyzed the secretion of a cytokine from the type I interferons family, IFN-I, which is known to be an antiviral modulator by activating ISGs (interferon-stimulated genes) (Lang et al., 2022; McDougal et al., 2022). In fish, several ISGs have been characterized (Xu et al., 2015), and the antiviral activity and protection of IFN-I have been studied (O'Farrell et al., 2002; L. I. Wang et al., 2006; Svingerud et al., 2012). Despite the increased levels of IFN-I in all cell lines at 14°C in infected groups compared to control groups, an up-regulation of MHC-I molecules was not observed, as mentioned previously in this section. These results suggest that the IFN-I signaling pathway was not effective in preventing antigen presentation disruption at 14°C or that other temperature-sensitive factors are impaired affecting the protective role of IFN-I (Lane et al., 2018).

Studies have shown that the antiviral responses of type I interferons is temperature-sensitive resulting in reduced antiviral responses involving impairment of ISGs (Lane et al., 2018). This supports the idea than even though IFN-I was produced by rainbow trout cell lines, the antiviral responses to avoid viral interference in the antigen presentation pathway were not efficient. Interestingly, RTGut-GC showed modest IFN-I levels at 14°C, despite the fact that it did not have significant changes in the antigen processing and presentation pathway, suggesting that intestinal epithelial cells may have a distinct regulatory mechanism (Konjar et al., 2017; Segrist & Cherry, 2020).

In summary, this chapter demonstrate that moderate suboptimal temperatures impairs different steps of the antigen presentation pathway in VHSV-infected rainbow trout cell lines, with a noticeable difference between cell-lines. These disruptions included the decrease in MHC/ β 2m surface expression and increased β 2m release, suggesting a blocking in MHC-I folding, recycling and/or degradation together with a possible disruption in peptide loading. These results offer a molecular insight into how suboptimal temperatures can compromise antigen presentation and cellular immunity in fish.

Chapter 4: Determining T cell activation using VHSV-infected Rainbow trout (*Oncorhynchus mykiss*) dorsal fin cells under suboptimal temperatures

4.1 Overview

Antigen presentation and cytotoxic T cell responses have a key role in fighting intracellular infections and are well-studied in mammals, however, their functions in teleost fish are poorly understood. In this study, an antigen presentation assay was developed using rainbow trout dorsal fin cells to understand antigen-specific cytotoxic responses in rainbow trout at different temperatures. Dorsal fin cells were exposed to heat-inactivated viral hemorrhagic septicemia virus (VHSV) IVb and then co-cultured with splenocytes from trout previously injected with the same heat-inactivated VHSV. Cytotoxicity was analyzed using a lactate dehydrogenase (LDH) release assay, and cell surface expression of different immune markers were evaluated by flow cytometry. Results showed that dorsal fin cells expressed high levels of MHC-I and MHC-II α , and low CD3 ϵ and IgM levels, indicating active antigen presentation functions. Co-cultures of VHSV-stimulated fin cells with activated splenocytes resulted in significantly increased LDH release at 12 hours, compared to control groups. In addition, splenocytes from VHSV-injected fish had stronger cytotoxic responses compared to naïve fish, suggesting an antigen-specific response. To evaluate the role of MHC-I in this response, an anti-MHC-I antibody was used to block MHC-I molecules on dorsal fin cells before the co-culture. This blockade decreased LDH release, confirming the contribution of MHC-I in antigen-specific recognition. Finally, to investigate the effect of suboptimal temperatures during antigen recognition, fin cells were exposed to heat-inactivated VHSV IVb and then co-culture with activated splenocytes at 10, 16 or 22°C, resulting in a decreased cytotoxic activity at 10°C compared to higher temperatures. These results indicate that activated splenocytes against VHSV IVb have a higher cytotoxic activity, probably due to recognition of viral peptides presented on MHC-I molecules on the cell surface of dorsal fin cells.

In addition, this recognition is impaired at lower temperatures providing crucial knowledge on the effect of suboptimal temperatures during antigen presentation and recognition. Moreover, dorsal fin cells show strong potential as antigen-presenting cells and offer a useful tool for studying antigen presentation and T cell activation in response to pathogens or vaccine candidates.

4.2 Introduction

Aquaculture is one of the fastest-growing industries in global food production and is projected to supply approximately 60% of all seafood consumed worldwide by 2030. However, the industry faces significant challenges from disease outbreaks, which results in estimated economic losses of 6 billion USD annually (Brummett & Anderson, 2020; FAO, 2024). One of the deadliest infectious diseases in aquaculture is caused by the viral hemorrhagic septicemia virus (VHSV). This virus causes severe disease outbreaks, high mortality rates, and can infect several species such as rainbow trout (Escobar et al., 2018; Skall et al., 2005). Phylogenetic analyses have identified four genotypes of VHSV, being the genotype IVb present in the Great Lakes region (Pierce & Stepien, 2012b). Several researchers have produced vaccines to control disease outbreaks generated by this virus; however, vaccines offer moderate protection (Kole et al., 2022; Nishizawa et al., 2011; Vinay et al., 2013). Therefore, a better understanding of the fish immune system and host-pathogen interactions is essential to develop more effective tools against VHSV.

The antigen presentation pathway is used by the immune system to detect and respond to viral infections. This process happens within the antigen-presenting cells (APCs) which are categorized into professional and non-professional APCs depending on their surface markers and

functions. All nucleated cells express the major histocompatibility complex class I (MHC-I) molecules, but only professional APCs express MHC class II (MHC-II) and key co-stimulatory molecules such as CD80/86, CD83, CD40 (Hamilos, 1989; Sprent, 1995). Among these, the MHC-I receptor key during the presentation of viral peptides to CD8⁺ T cells and by initiating the adaptive immune responses (Croft et al., 2019; ; Smyth et al., 2012). During a viral infection, viral proteins are produced within infected APCs and then processed by the ubiquitin-proteasome system into smaller peptides. These peptides are then translocated into the endoplasmic reticulum (ER) where they are loaded onto the complete MHC-I/ β 2-microglobulin (β 2m) receptors with the assistance of several chaperones, for example tapasin. Once peptide loading is complete, the MHC-I-peptide complex is transported to the cell surface (Pishesha et al., 2022). At the cell surface, this complex is recognized by CD8⁺ T cells by their T cell receptor (TCR) and the CD8 co-receptor (Armstrong et al., 2008; Szeto et al., 2021). This recognition produces the activation of CD8⁺ T cells, leading to the expression and release of several killing effectors such as perforin and granzyme which induce apoptosis and cell death of the infected APC. In addition, activated T cells secrete cytokines such as interleukin 2 (IL2), interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α), which then helps to activate the immune system to clear the viral infection (Cox et al., 2013; Remakus & Sigal, 2011; J. Yang et al., 2019). The T cell receptor (TCR) is restricted to recognizing self-MHC molecules, meaning that a specific immune response is only generated when a viral peptide is presented by cells of the same host. When a viral peptide is recognized on self-MHC molecules, the T cells respond in a specific manner. However, when viral peptide is presented by non-self MHC, the immune response becomes less specific. In this case, non-self MHC may trigger a broader, non-specific immune response not only against the viral infection but

also against any cell expressing non-self MHC (Garboczi & Biddison, 1999; La Gruta et al., 2018; Zareie et al., 2020).

The study of the antigen presentation pathway in rainbow trout began around 30 years ago, initially focusing on cloning and characterizing MHC genes to understand their structure and function. In terms of antigen-presenting cells in fish, studies have shown that dendritic cells (DCs), macrophages and B cells are present in various tissues, having different functions. DCs are found in kidney, spleen and mucosal tissues such as skin and gills, where they present phagocytic capacities and express markers such as MHC-II, CD80/86, CD83 and surprisingly CD8 α (Shao et al., 2015; Soleto et al., 2018, 2019). In addition, studies have shown that fish macrophages (Cuesta et al., 2006; Hodgkinson et al., 2015; Rougeot et al., 2019) and B cells (Miller et al., 1998; Wu et al., 2020a, 2020b) are also phagocytic and involved in bactericidal activity against a variety of pathogens. In addition to these classical antigen-presenting cells, non-typical cells have also been shown to have the potential to act as APCs. For instance, a recent study demonstrated that fin cells express key markers involved in antigen presentation including MHC-I, MHC-II and classical professional APCs markers, such as CD83, CD205, and CSF1R (Vo et al., 2025). While these studies give valuable insight into the expression of antigen presentation markers and chaperone proteins at the transcript and protein levels, little is known about the functional aspects of the presentation of viral peptides on MHC-I to further activate T cells, and how this recognition is affected by temperature fluctuations. This represents a crucial gap in our understanding of the immune functions of this pathway in rainbow trout exposed to suboptimal temperatures.

Developing an antigen presentation assay is a key tool to better understand the immune process involved in fish responses to viral infections during temperature fluctuations events.

However, this task is not easy. As mentioned earlier, there is still a huge knowledge gap in understanding the antigen presentation pathway in fish. The function of CD8⁺ T cells in fish is not fully understood, particularly if they can recognize viral peptides presented by MHC-I molecules. In addition, the function of antigen-presenting cells (APCs) is not fully characterized during a viral infection, or the function of key molecules expressed and secreted in cytotoxic responses such as perforin, granzymes and cytokines including IFN γ , TNF α , and IL-2. In fact, a lot of aspects of this process remain a mystery. To clarify this process, an antigen presentation assay was developed. One of the major problems in studying antigen presentation in fish is the lack of genetically identical individuals or cell lines from the host. To solve this, fin cells were selected as target cells, because they can be clipped non-lethally and have shown potential to function as antigen-presenting cells (Vo et al., 2025). Thus, VHSV was used as infection model and CD8⁺ T cells were isolated from the spleen of heat-inactivated VHSV-injected fish. To evaluate cytotoxic responses, cell death was measured by the release of lactate dehydrogenase (LDH). Developing this type of antigen presentation assay represents a crucial step toward understanding the functional immune response in fish against pathogens during climate change events and could serve as a foundation for future vaccine development and scheduling.

4.3 Materials and methods

4.3.1 Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*) of 100 $\pm$ 5 g were obtained from Lyndon hatcheries, Ontario, Canada. Fish were kept in 122 L tanks in the Waterloo Aquatic Threats in

Environmental Research Facility at the University of Waterloo, Ontario, Canada. Fish were maintained at 6.55 g/L density at 12°C and fed at 1% of their body weight every other day. Parameters such as pH, dissolved oxygen and ammonia were checked daily.

4.3.2 Injection with heat-inactivated VHSV IVb

For experimental procedures, fish were separated into 66 L tanks at a biomass of 9.14 g/L and acclimated for 14 days prior to infection. Fish were pit tagged intramuscularly (i.m.) and dorsal fins were extracted for posterior cell culture from fin explants (see below). Fish were then injected intraperitoneally (i.p.) twice seven days apart with 100 µL of heat-inactivated VHSV IVb (0.5×10^6 pfu/mL) in L-15 medium to obtain VHSV-activated splenocytes or with L-15 medium alone to obtain naïve splenocytes (Figure 4.1). After 14 days post-injection with inactivated virus, fish were euthanized with MS-222 in sodium bicarbonate buffer and splenocytes were isolated (see below) for further antigen presentation assay.

4.3.3 Fin cell culture

Fin cell cultures were collected as described by (Vo et al., 2025) with minimal modifications. Briefly, dorsal fins were cut into small pieces and placed in 6-well plates containing 2 mL of complete media (Leibovitz's L-15 Medium; L-15, 20% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), 2 µg/mL fungizone, 10 mM HEPES and 40 µM beta-mercaptoethanol). Media was discarded and fresh media was added every 5 days until the antigen presentation assay. Cells were held at 16°C in the dark.

4.3.4 Splenocyte extraction

Spleens were aseptically removed and passed through a 70 μ m cell strainer with L-15 supplemented with 10% FBS and 1% P/S. The cell suspension was centrifuged at 400 $\times g$ for 5 min at 4°C, the supernatant was removed, and cells were resuspended in supplemented L-15 media. Hypotonic lysis was performed to eliminate red blood cells by adding 8 mL of distilled water for 10 s after which PBS 10X was added to stop the reaction (Hu et al., 2018). Cells were centrifuged as described previously, and the cell pellet was resuspended in supplemented L-15 media to count the total number of cells per milliliter using a hemocytometer with trypan blue staining.

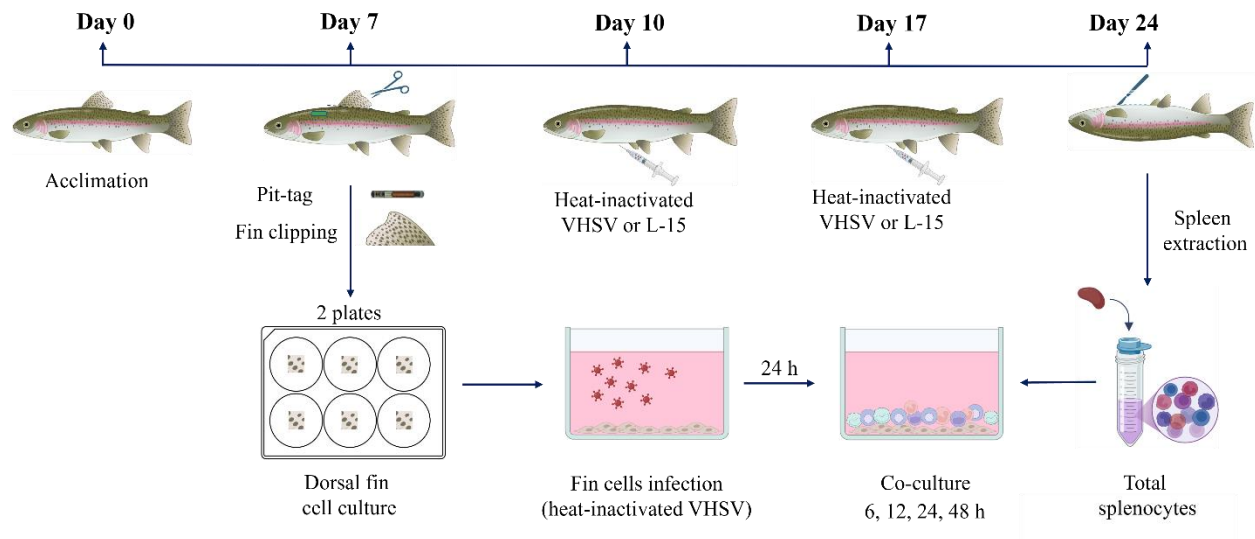


Figure 4.1. Experimental design for the antigen presentation assay.

4.3.5 Viral propagation

Epithelioma papulosum cyprini (EPC) cells were used to propagate viral hemorrhagic septicemia virus (VHSV) IVb (CEFAS strain U13653) as described by (Abram et al., 2019).

VHSV IVb was diluted in infection media (L-15 supplemented with 2% FBS) to infect EPC cells. After 2 hours, the media was replaced with fresh media and infection was continued for 10 days. Virus-containing media was filtered through a 0.22 μ m filter and stored at -80°C for future use. The TCID₅₀/mL and pfu/mL were determined using the Poisson distribution. For heat inactivation, VHSV IVb aliquots were placed in a dry bath incubator at 56°C for 30 min with constant agitation (Tafalla et al., 2008). Inactivation was confirmed by inoculating EPC cells and analyzing the lack of cytopathic effect after 10 days of infection.

4.3.6 Flow cytometry analysis

The cell surface expression of antigen presentation, T cell and B cell markers in splenocytes and dorsal fin cells were evaluated by flow cytometry. Briefly, splenocytes and fin cells (0.5×10^6 cells), were incubated with FACS buffer (PBS 1x supplemented with 2% FBS) for 10 min at 4°C to block nonspecific binding. Following incubation, cells were centrifuged at 400 $\times g$, for 5 min at 4°C and resuspended in 100 μ l of the following primary antibody: chicken anti-trout MHC-I (20 ng/ μ l), chicken anti-trout MHC-II α (6.25 ng/ μ l), mouse anti-CD3 ϵ (hybridoma supernatant) and mouse FITC-anti-salmonid IgM (1:100, Cedarlane Lab, #CLF004F). Cells were incubated for 1 h at 4°C and washed with FACS buffer at 400 $\times g$ for 5 min at 4°C, to then incubate with 100 μ l of the secondary antibodies AlexaFluor™647 Goat anti-chicken IgY or anti-mouse IgG (1:600, ThermoFisher, #A-21449/ #A-21235) for 30 min at 4°C. Finally, cells were washed and resuspended in FACS buffer to analyze the cell surface expression of each marker by flow cytometry using a BD FACS Aria Fusion flow cytometer with FACSDiva software. Cell viability was assessed by propidium iodide (1 μ g/mL), and live cells were gated to analyze the percentage of positive cells. Unstained cells were used as autofluorescence control. The purified chicken IgY (BioLegend,

#402101), FITC-mouse IgG2a (Cedarlane Lab, #CLCMG2A01) and mouse IgG (ThermoFisher, #31903) isotype controls were used at the same concentration of the primary antibodies.

4.3.7 Antigen presentation assay

Twenty-four hours before the antigen presentation assay, dorsal fin cells were exposed to heat-inactivated VHSV IVb (MOI 5) for 24 h at 16°C in infection media to allow viral peptides to be presented by MHC molecules. After 24 hours, media was replaced by L-15 supplemented with 10% FBS and 1% P/S and splenocytes from VHSV-sensitized fish were co-cultured with dorsal fin cells in a ratio of 10 : 1 (splenocytes : fin cells) for 6, 12, 24 and 48 at 16°C. At each time point, media and cells were collected for further analysis. Several control groups were used to analyze the antigen presentation: fin cells alone (group 1: spontaneous lysis), fin cells exposed to heat-inactivated VHSV IVb (group 2: control), fin cells co-cultured with splenocytes (group 3: non-specific lysis), fin cells exposed to heat-inactivated VHSV IVb and co-cultured with splenocytes (group 4: specific lysis), and fin cells incubated with lysis buffer (group 5: lysis 100%). In addition, splenocytes alone were cultured during specific the same time points (group 6). To analyze the effect of temperature in the T cell activation, cells were co-cultured at 10°C, 16°C or 22°C using the same methodology mentioned previously.

4.3.8 Lactate dehydrogenase (LDH) measurements

After the antigen presentation assay samples were collected, cell media was collected and centrifuged at 400 $\times g$ for 5 at 4°C. The supernatant was recovered to measure cell death by the release of lactate dehydrogenase (LDH) using the CyQUANT™ LDH Cytotoxicity Assay (ThermoFisher, #C20301). Briefly, 50 μ L of supernatant was added to a 96-well plate in triplicate,

together with media alone as negative control and positive control provided by the manufacturer. In addition, a 100% lysis control group was obtained by incubating fin cells with lysis buffer for 45 min at 16°C and media was collected, centrifuged at 400 xg for 5 min at 4°C to then add 50 µL per well to the 96-well plate. Then, the plate was incubated at 37°C for 30 min and the read at 490 nm/680 nm. Percentage of cytotoxicity was calculated using the following formula:

$$\% \text{ Cytotoxicity} = \left(\frac{\text{Control/Non-specific/Specific lysis} - \text{Spontaneous lysis}}{\text{Lysis 100\%} - \text{Spontaneous lysis}} \right) \times 100$$

4.3.9 MHC-I blockade

Fin cells were infected with heat-inactivated VHSV as described above, and the media was replaced with a chicken anti-trout MHC-I antibody diluted in infection media (35 ng/µl). Cells were incubated for 2 h at 16°C to block the MHC-I molecules on the cell surface of the VHSV-infected fin cells. After incubation, media was changed and infected fin cells were co-cultured with VHSV-activated splenocytes for 12 h at 16°C using an effector : target ratio of 10:1. Following incubation, cell media was collected to analyze the LDH release as described previously.

4.3.10 Western blot analysis

Also, after the co-culture, the supernatant was recovered as mentioned and the cell pellet containing splenocytes was used to perform analysis on total protein extracts, in addition to the adherent fin cells. For protein extracts, cells (splenocytes or fin cells) were washed with ice-cold PBS and then incubated with RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40

Substitute, 0.25% Sodium Deoxycholate) for 15 min on ice. Then cells were resuspended (splenocytes) or scraped (fin cells) and centrifuged at 14,000 xg for 20 min at 4°C. Supernatants containing total proteins were quantified using a BCA kit following manufacturer's instructions and then stored at -80°C for further use. The expression of β 2-microglobulin, tapasin and perforin was analyzed by western blot. Briefly, proteins were separated using 12% (tapasin and perforin) or 15% (β -microglobulin) polyacrylamide gel and transferred to nitrocellulose membrane. Unspecific binding was blocked by incubating the blots with skim-milk 5% in TBS-T for 1 h at room temperature. Then, membranes were incubated with affinity purified rabbit anti-trout β 2m (3 μ g/mL), rabbit anti-trout tapasin (3 μ g/mL) or rabbit anti-human perforin (1 μ g/mL; PA5-79865, ThermoFisher), in addition to the loading control rabbit anti β -actin antibody (1:10,000, Sigma) in blocking solution overnight at 4°C. Membranes were washed three times with TBS-T for 10 min at room temperature. Then, blots were incubated with donkey anti-rabbit HRP secondary antibody (1:50,000, Sigma) in blocking solution for 1 h at room temperature. Bands were detected using Peroxide/luminol solution (Clarity Western ECL Substrate, BioRad). Protein expression was standardized related to β -actin and then normalized to the spontaneous lysis control.

4.3.11 Sandwich ELISA

Supernatant were used to quantify extracellular expression of IFN γ and IL-2 by sandwich ELISA. Briefly, ninety-six microtiter flat bottom plates (Immulon 4HBX, Fisher Scientific) were coated with 100 μ L/well of goat anti-trout IFN- γ , or goat anti-trout IL-2 capture antibody (Cedarlane Lab) at 1 μ g/mL or 2 μ g/mL, respectively, in coating buffer (sodium carbonate, sodium bicarbonate, pH 9.6) and incubated for 4 hours at room temperature. Plates were then blocked with 300 μ L/well of blocking solution and incubated for 1 hour at 37°C. Then, wells were incubated at

4°C overnight with 100 μ L of cell media supernatants or recombinant trout IFN- γ or IL-2 serially diluted in L-15 2% FBS with concentrations ranging from 800 pg/mL to 12.5 pg/mL for IFN- γ and 400 pg/mL to 12.5 pg/mL for IL-2. The next day, 100 μ L of chicken anti-trout IFN- γ (1 μ g/mL) or rabbit anti-trout IL-2 (1 μ g/mL) detection antibody (Cedarlane Lab) was added to each well and plates were incubated for 3 hours at room temperature. Then, plates were incubated with 100 μ L/well of anti-chicken IgY (1:4000) or anti-rabbit IgG (1:1000) conjugated to biotin (Cedarlane Lab), followed by incubation with 100 μ L/well of streptavidin conjugated HRP (Cedarlane Lab) diluted to 1:1000 for 1 hour at room temperature in the dark. Plates were then washed four times and incubated with 100 μ L/well of TMB Plus 2 (Cedarlane Lab) at room temperature in the dark for 30 minutes, at which point 100 μ L/well of stop solution was added. Absorbance was measured at 450 nm on a Synergy H1 Hybrid Reader (BioTek Instruments).

All samples were measured in triplicate, and all antibodies were diluted with 5% skim milk in TBS-T. Plates were washed four times with TBS-T between each incubation period unless stated otherwise. Serial dilutions of recombinant trout IFN- γ or IL-2 on each plate were used to plot a standard curve (Supplementary Figure S.3 and Figure S.4, respectively), and interpolation of the linear trend line equation was used to calculate the concentration of IFN- γ or IL-2 in each sample.

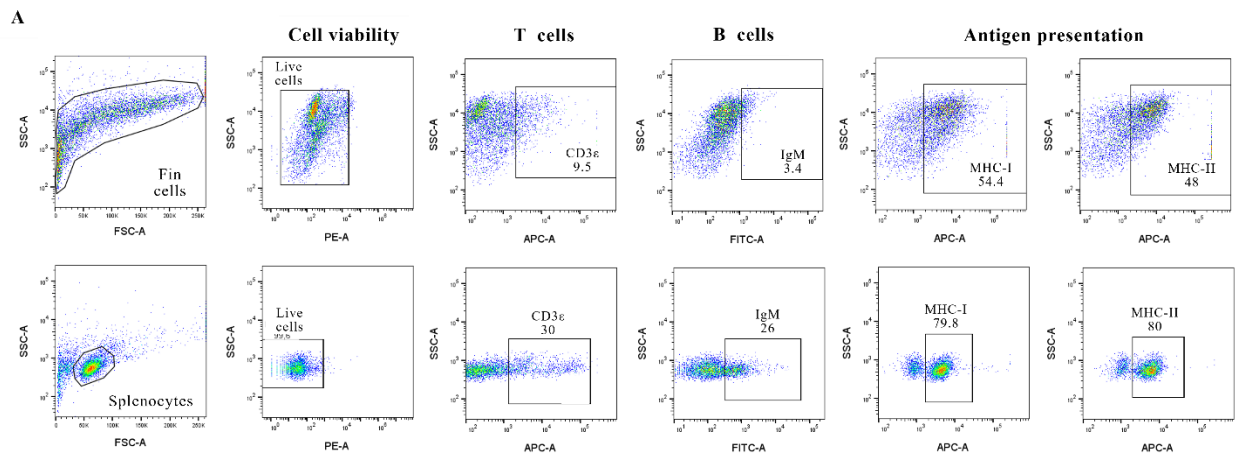
4.3.12 Statistical analysis

Data was graphed and statically analyzed with the GraphPad Prism 10 software. Significant differences between each group at each time point were determined using One-way or 2-way ANOVA with Tukey's multiple comparison test.

4.4 Results

4.4.1 Expression of immune markers in dorsal fin cells

To compare the cellular composition of splenocytes and dorsal fin cell cultures, flow cytometry was performed to assess the surface expression of key immune markers. These included antigen presentation molecules (MHC-I, MHC-II α), a T cell marker (CD3 ϵ) and a B cells marker (IgM). Both splenocytes and dorsal fin-derived cells were analyzed for the percentage of cells expressing these cell surface proteins. Representative flow cytometry plots are shown in Figure 4.2A, illustrating the gating strategy for both dorsal fin cells and splenocytes. Cell viability was assessed using propidium iodide, and only live cells were included in the analysis. From the gated live cell population, the percentage of cells expressing each surface marker was quantified. Dorsal fin cells expressed MHC-I (60%) and MHC-II α (50%), indicating the presence of antigen-presenting capabilities. In terms of lymphocyte markers, approximately 20% of dorsal fin cells were positive for CD3 ϵ , identifying them as T cells, while 10% were IgM-positive B cells. When compared to splenocytes, dorsal fin cells exhibited lower expression levels for all assessed surface markers (Figure 4.2B).



B

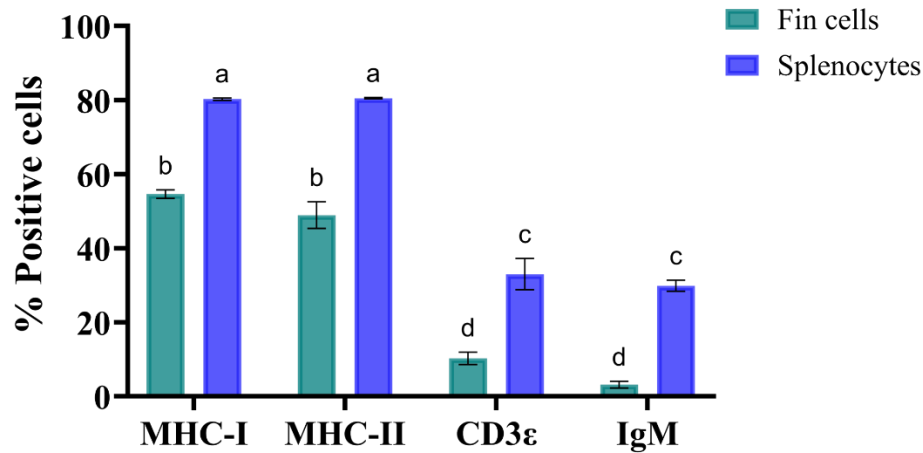


Figure 4.2. Flow cytometry analysis of immune cell markers. Fin cells and splenocytes were labeled with anti-CD3ε, anti-MHC-I, anti-MHC-IIα and FITC-conjugated anti-IgM antibodies. Propidium iodide stain (PI) was used to exclude non-viable cells, and analysis was performed on the live cell population (PI-negative). (A) Representative flow cytometry plots showing forward scatter (FSC) vs side scatter (SSC) to distinguish cell populations. (B) Quantification of specific immune cell subsets. Data represent mean $\pm$ standard error from four independent replicates.

4.4.2 Kinetics of cytotoxic activity against dorsal fin cells

To evaluate the time-dependent cytotoxic response of VHSV-activated splenocytes, dorsal fin cells were used as target cells. Cytotoxic activity was evaluated at 6-, 12-, 24-, and 48-hours following co-culture between VHSV-activated splenocytes and VHSV-infected dorsal fin cells. At each time point, the percentage of cell lysis was quantified by measuring lactate dehydrogenase

(LDH) release, serving as an indicator of target cell membrane damage and cytotoxicity. An increase in cell lysis was observed at 12 hours of co-culture compared to control group, followed by a decline at 24 and 48 hours (Figure 4.3).

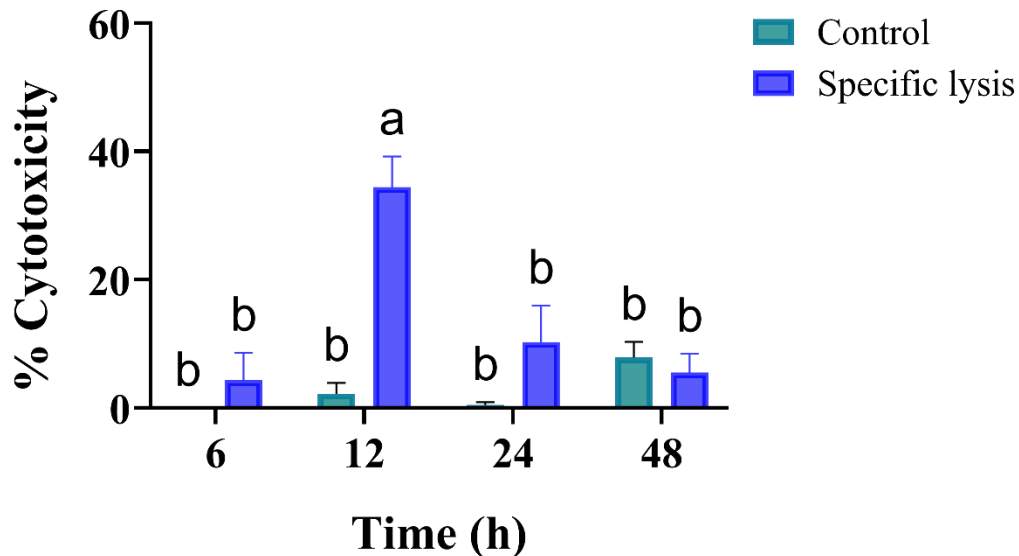


Figure 4.3. Cytotoxic activity of activated splenocytes against VHSV-infected fin cells. VHSV-Infected fin cells were co-cultured with activated splenocytes for 6, 12, 24, or 48 hrs at 16°C. Cell media was collected at each time point, and lactate dehydrogenase (LDH) release was quantified using the CyQUANT™ LDH Cytotoxicity Assay Kit. Data represent mean $\pm$ standard error from six independent replicates.

4.4.3 Cytotoxic activity of naïve versus activated cells

To compare the cytotoxic potential of naïve and VHSV-activated splenocytes, co-culture assays were performed using VHSV-infected dorsal fin cells as targets. LDH release was measured

after 12 hours of co-culture. As shown in Figure 4.4, VHSV-activated splenocytes exhibited significantly higher cytotoxic response compared to control group and naïve splenocytes, as indicated by a greater percentage of cell lysis. Naïve splenocytes showed minimal cytotoxicity, with LDH levels comparable to background control conditions.

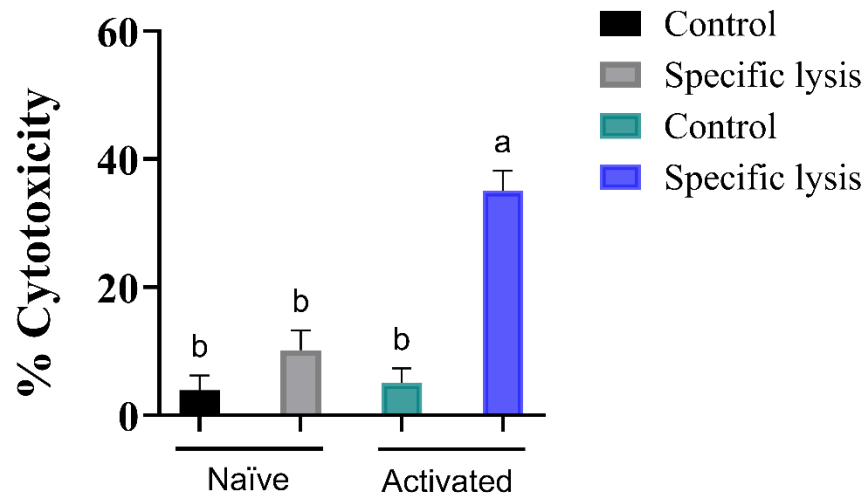


Figure 4.4. Cytotoxic activity of naïve compared to activated splenocytes against VHSV-infected fin cells. Rainbow trout was injected with L-15 or heat-killed VHSV. Then, VHSV-Infected fin cells were co-cultured with activated splenocytes, at an effector : target ratio of 10:1, for 12 hrs at 18°C. Cell media was collected, and lactate dehydrogenase (LDH) release was quantified using the CyQUANT™ LDH Cytotoxicity Assay Kit. Data represent mean $\pm$ standard error from six independent replicates.

4.4.4 MHC-I blockade decrease the cytotoxic response from VHSV-activated splenocytes

To determine whether the cytotoxic response was mediated by CD8⁺ T cells, MHC-I molecules on dorsal fin cells were blocked using an anti-MHC-I antibody. Following this

treatment, dorsal fin cells were co-cultured with VHSV-activated splenocytes, and LDH release was measured. As shown in Figure 4.5, a significant decrease in cell lysis was observed in the MHC-I blocked group, resulting in 15% cytotoxicity, compared to the non-blocked group, showing 35% cell lysis (Figure 4.5).

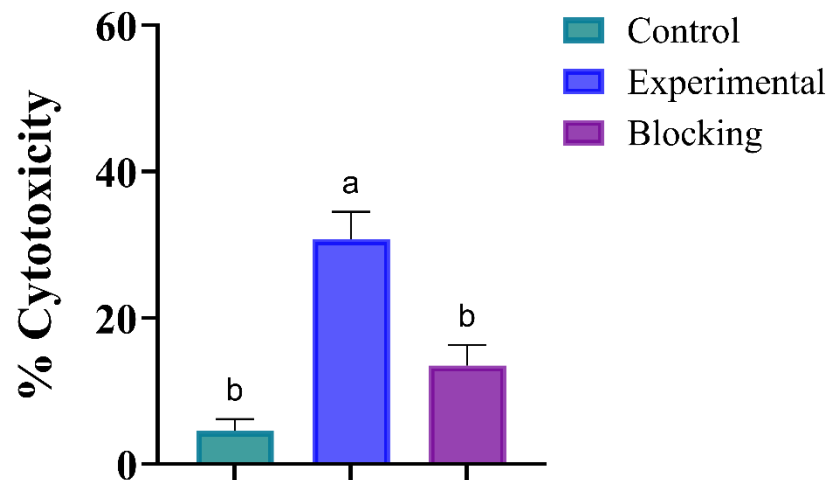
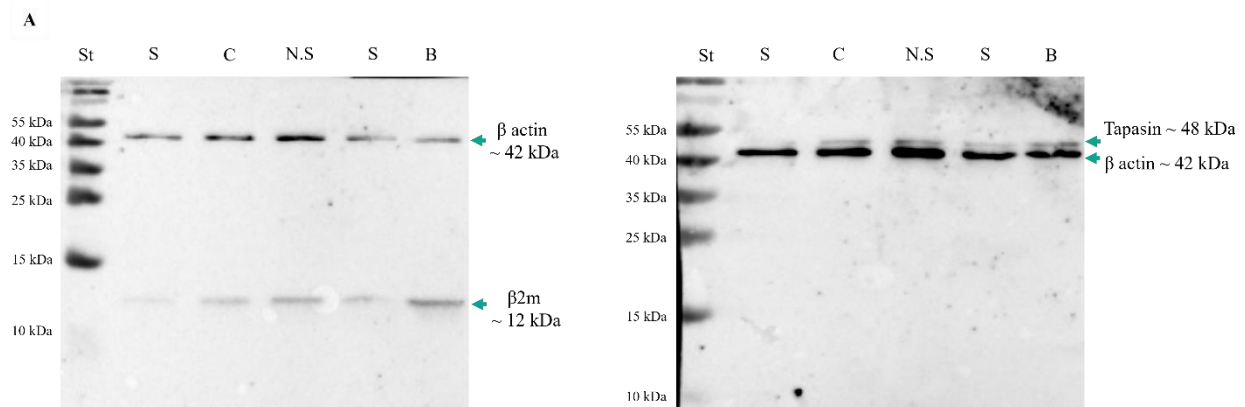


Figure 4.5. Cytotoxic activity of activated splenocytes against VHSV-infected fin cells after blockade of MHC-I. VHSV-Infected fin cells were pre-incubated with a chicken anti-MHC-I antibody for 2 hrs 16°C. Following incubation, cells were co-cultured with activated splenocytes, at an effector-to-target ratio of 10:1 for 12 hrs at 16°C. Cell media was collected, and lactate dehydrogenase (LDH) release was quantified using the CyQUANT™ LDH Cytotoxicity Assay Kit. Data represent mean $\pm$ standard error from six independent replicates.

4.4.5 Regulation of β 2-microglobulin and tapasin

The stability and optimal loading of high-affinity peptides onto MHC-I molecules depends on the presence of β 2-microglobulin and function of several chaperones including tapasin. To evaluate whether the expression of these proteins is regulated during viral antigen presentation, we measured their protein levels by Western blot. Dorsal fin cells exposed to heat-inactivated VHSV were co-cultured with VHSV-activated and total protein extracts were prepared to analyze β 2-microglobulin and tapasin expression. β -actin was used as a loading control. Comparison between the control group, the non-blocked group and the MHC-I blocked group revealed no significant differences in the expression levels of these chaperones (Figure 4.6).



B

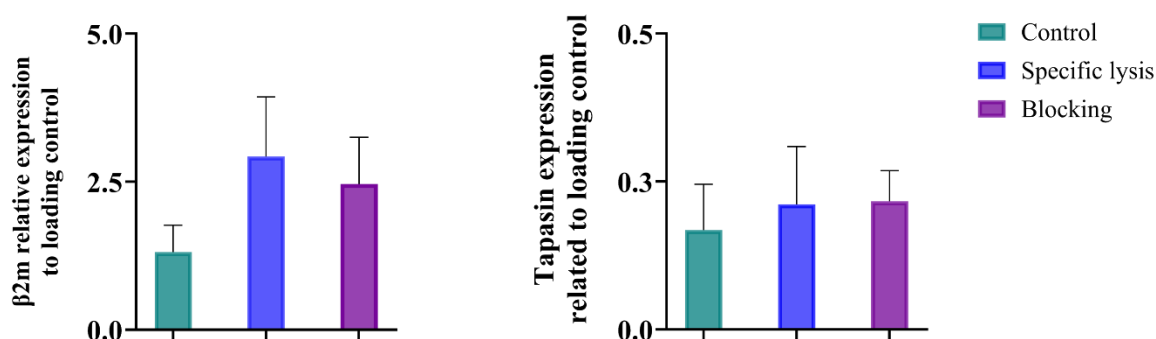


Figure 4.6. Protein expression of β 2-microglobulin and tapasin. VHSV-Infected fin cells were co-cultured with activated splenocytes, for 12 hrs at 16°C. The expression levels of β 2m and tapasin were analyzed by western blot using polyclonal rabbit anti- β 2m and anti-tapasin antibodies. (A) Representative blots show the expression of β 2m, tapasin and the loading control β -actin. Page Ruler molecular weight marker was used as reference. (B) Quantification of β 2m and tapasin protein levels relative to β -actin expression. Data represent mean $\pm$ standard error from six independent replicates.

4.4.6 Cytokine secretion during cytotoxic response

The secretion levels of IFN γ and IL-2 were evaluated after 12 hours of co-culture of heat-killed VHSV-exposed fin cells with activated splenocytes using sandwich ELISA. Standard curves were generated using recombinant rainbow trout IFN γ and IL-2 proteins to quantify cytokine concentration in the cell culture supernatants (Supplementary Figure S.3 and Figure S.4, respectively). However, cytokine levels were below the detection limit of the assay in all samples (data not shown).

4.4.7 Perforin expression as killing effector mechanism

Western blot analysis was performed to analyze the expression of perforin both intracellularly and in the cell culture media of VHSV-exposed fin cells co-cultured with activated splenocytes after the blockade of MHC-I molecules. Due to the lack of a trout-specific perforin antibody, a rabbit anti-human perforin antibody was used; however, no cross-reactivity was detected in the samples (data not shown).

4.4.8 Effect of suboptimal temperatures in the activation of T cells

To analyze how temperature affects the recognition of viral peptides presented on MHC-I molecules by CD8⁺ T cells, dorsal fin cells were exposed to heat-inactivated VHSV IVb and then co-cultured with activated splenocytes. During the co-culture, cells were exposed to 10°C, 16°C and 22°C and the cell death was evaluated by LDH release. In Figure 4.7 it can be observed that there is higher cytotoxic activity when cells were exposed to 16°C and 22°C compared to 10°C.

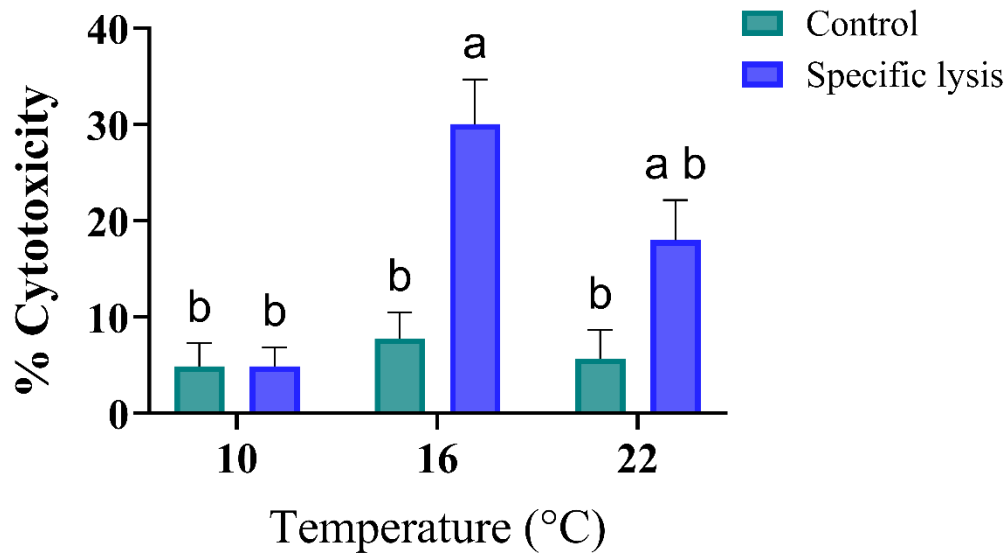


Figure 4.7. Cytotoxic activity of activated splenocytes against VHSV-exposed fin cells under different temperatures. VHSV-Infected fin cells were co-cultured with activated splenocytes, at an effector-to-target ratio of 10:1 for 12 hrs at different temperatures: 10°C, 16°C and 22°C. Cell media was collected, and lactate dehydrogenase (LDH) release was quantified using the CyQUANT™ LDH Cytotoxicity Assay Kit. Data represent mean $\pm$ standard error from six independent replicates.

4.5 Discussion

As mentioned, temperature is a key modulator of immune responses and viral infections, therefore, it is crucial to understand how suboptimal temperatures can affect the host-pathogen interactions. It is clear that antigen processing is key for activating later adaptive immune responses such as the induction of CD8⁺ T cell responses. However, little is known about the

interaction between antigen presenting cells (APCs) and CD8⁺ T cells through MHC-I molecules in rainbow trout. Moreover, there is a lack of knowledge on how suboptimal temperatures could affect the recognition of viral peptides presented on MHC-I by CD8⁺ T cells. For this reason, this chapter evaluated the effect of suboptimal temperatures during the presentation of VHSV IVb peptides by MHC-I molecules to CD8⁺ T cells. The main challenges were: 1) antigen presenting cells (APCs) are not fully characterized in rainbow trout, 2) unavailability of isogenic (genetically identical) rainbow trout strains and/or cell lines to allow a genetic match between MHC-I molecules and T cell receptors and 3) a standardized protocol to analyze MHC-I/CD8⁺ T cell recognition and T cell activation. To overcome these challenges, dorsal fin cells were used as potential antigen presenting cells (Vo et al., 2025) and from those fish, total splenocytes previously activated against VHSV IVb, were used to analyze the presentation of viral peptides to CD8⁺ T cells from the spleen (Figure 4.1). Before elucidating the effects of suboptimal temperatures during antigen recognition, it was necessary to standardize the antigen presentation assay using dorsal fin cells. To evaluate antigen recognition by CD8⁺ T cells the cell death of the dorsal fin cells was analyzed by the release of lactate dehydrogenase (LDH). In addition, T cell activation was evaluated by quantifying the secretion of key cytokines like IFN γ and IL-2.

First, the cell surface expression of key molecules during the activation of the adaptive immune system were evaluated in dorsal fin cells and splenocytes (Figure 4.2). Flow cytometry analysis demonstrated that dorsal fin cells expressed both MHC-I (60%) and MHC-II α (50%) which are key molecules for antigen presentation (Pishesha et al., 2022). It is important to highlight that MHC-II α is a professional antigen presenting cell marker, supporting the idea that fin cells could act as antigen presenting cells in rainbow trout (Vo et al., 2025). On the other hand, even

though the expression of CD3 ϵ (T cells) and IgM (B cells) was significantly lower than in splenocytes, expression of these molecules suggests that dorsal fin tissue may have resident immune cells that contribute to local immune responses against pathogens such as lymphocytes (Castranova et al., 2024).

The kinetics of cytotoxic activity from VHSV-activated splenocytes against dorsal fin cells exposed to VHSV IVb showed a clear peak at 12 hours post co-culture (Figure 4.3). However, there was a sharp decline in cytotoxicity between 24 and 48 hours that could be attributed to T cell exhaustion where there is a decline in cellular function due to persistent exposure to the antigen thus reducing their cytotoxic ability (Spencer et al., 2018; Wherry & Kurachi, 2015; Zhang et al., 2023). During this process several regulatory mechanisms are activated by secreting cytokines such as IL-10, TGF- β and IL-2, among others (Zhang et al., 2023; Zhu et al., 2023). The kinetic behavior in mammals is rapid and elevated, showing specific lysis of 80% detectable as early as 2-4 hours post-stimulation (Wolint et al., 2004; Zöphel et al., 2022). In contrast, the cytotoxic response observed in this chapter suggests that the molecular mechanisms of antigen recognition and cytotoxic activation in fish may be slower than in mammals, as has been mentioned in several studies (Alexander & Ingram, 1992; Ellis, 2001; Kordon et al., 2018). The cytotoxic response after 12 hours suggests an activation of splenocytes against VHSV IVb *in vivo* during fish injections and subsequently *in vitro* recognition of VHSV peptides on MHC-I presented by dorsal fin cells. Several studies have demonstrated virus-specific activation of leukocytes in different fish species including rainbow trout (Utke et al., 2007), gibelio crucian carp (Somamoto et al., 2006, 2013), channel catfish (Hogan et al., 1996) and sea bass (García-Álvarez et al., 2024). Isogenic rainbow trout clone C25 was used to analyze the cytotoxic activity of peripheral blood leukocytes (PBL)

against the MHC-I-matched target cells RTG-2 following the infection with live VHSV (Utke et al., 2007). This study reported an increase in cytotoxic to VHSV infection, however, the incubation time during the co-culture was not specified (Utke et al., 2007). In carp, kidney leukocytes from clonal fish were used as effector cells which were co-cultured for 6 hours with the carp cell line CFS (Somamoto et al., 2006). The cytotoxic activity was analyzed using radioactivity and showed that after 6 hours of co-culture, the lysis reached 30-40% in infected cells versus the control groups (Somamoto et al., 2006). Years later, the same group evaluated the cytotoxic response from carp PBL against the same fin cell lines CFS within 6 hours, showing cytotoxic response not only from CD8⁺ T cells but also from B cells, and monocytes (Somamoto et al., 2013). Similarly, in channel catfish, the cytotoxic response of PBL against target cells was observed after 4 hours post co-culture using the release of chromium 51 (Hogan et al., 1996), while head kidney cells from sea bass showed cytotoxic activity resulting in 20% of lysed target cells using LDH release (García-Álvarez et al., 2024). While all these results support the existence of antigen-specific cell-mediated cytotoxicity in fish, methodological inconsistencies across studies complicate comparative analysis. Effector cells have been isolated from different sources, the duration of co-cultures varies between studies, and different detection methods to analyze cell death were used. Moreover, not all studies confirmed an MHC-I genetic match between effector and target cells. These limitations highlight the need to standardize protocols and genetically defined models to accurately understand T cell-mediated immunity in teleost fish. However, despite these differences, it is possible to find a consistent conclusion that teleost fish possess functional cytotoxic mechanisms capable of targeting virus-infected cells in an antigen-specific manner.

Importantly, activated splenocytes from VHSV-injected fish showed significantly higher cytotoxicity than naïve splenocytes, confirming an antigen-specific response against this virus (Figure 4.4). The observed increase in lactate hydrogenase (LDH) release after exposure to VHSV-exposed fin cells indicates that splenocytes actively and effectively recognized and lysed VHSV-exposed target cells. These results are supported by other researchers that have shown that it is necessary to sensitize an animal to obtain an effective and specific cell-mediated response from CD8⁺ T cells (Fischer et al., 2006; Nakanishi et al., 2011; Nakanishi & Okamoto, 1999; Somamoto et al., 2002, 2014; Toda et al., 2011). In mammals, when a viral infection is established in the host, the adaptive immune system is activated, resulting in the proliferation of virus-specific T cells. After the infection is cleared, most effector CD8⁺ T cells die but a subset differentiate in memory CD8⁺ T cells which are faster, stronger and more effective during a second infection (Turner et al., 2021). Immune cell memory hasn't been definitively proven in teleost fish (Stosik et al., 2021), for this reason many studies have used the strategy of immunizing animals two or three times to maintain the activated state of effector cells like CD8⁺ T cells (Somamoto et al., 2006, 2013; Toda et al., 2009, 2011; Utke et al., 2007).

While in Figure 4.4 it was observed that there was a possible antigen-specific response from activated splenocytes against VHSV-exposed fin cells, it is not clear if this response is due to the direct recognition of viral peptides loaded onto MHC-I molecules by CD8⁺ T cells since total splenocytes were used in this chapter. To measure cell-mediated cytotoxicity from CD8⁺ T cells it would be ideal to isolate this population from all other cells, however, this would require specific antibodies against CD8. Unfortunately, a specific antibody against rainbow trout CD8 was not available during this study, but as shown in previous chapters, an anti-trout MHC-I antibody

was available. Since the binding of the CD8 co-receptor to MHC-I molecules is essential during the recognition of viral peptides by CD8⁺ T cells (Chen et al., 2017; Devine et al., 2006), blocking the MHC-I molecules on dorsal fin cells before the co-culture with activated splenocytes using our anti-rainbow trout MHC-I antibody could demonstrate that this function is happening in this experiment. As shown in Figure 4.5, the significant reduction in cell lysis - from ~ 30% in non-blocked cells to ~ 15% in blocked cells – demonstrates the importance of MHC-I in the observed cytotoxic response. In mammals, MHC-I downregulation or blocking reduces CD8⁺ T cell-mediated killing, which is a common evasion mechanism utilized by pathogens and tumor cells (Koutsakos et al., 2019; Wu et al., 2025). While several studies have analyzed the levels of key modulators of the cell-mediated cytotoxic response in fish (García-Álvarez et al., 2024; Somamoto et al., 2006, 2013; Toda et al., 2009, 2011; Utke et al., 2007), this is the first study showing the direct contribution of MHC-I molecules during this process. An interesting point to mention is the involvement of natural killer (NK) cells during the cytotoxic response against VHSV-exposed dorsal fin cells. In mammals, natural killer cells are part of the innate immune response, eliminating virus-infected or tumoral cells by detecting the absence of MHC-I molecules on the cell surface (Mace, 2023). NK cells are activated when they recognize cells without MHC-I molecules, subsequently releasing cytotoxic granules similar to CD8⁺ T cells (Kumar. S, 2018; Moretta & Moretta, 2004). As mentioned previously, the results presented here demonstrate a decrease in cytotoxicity from activated splenocytes against VHSV-exposed fin cells, particularly when MHC-I molecules were blocked. It would be expected that the absence or reduction of self-MHC-I molecules on fin cells would lead to an increase in NK cell-mediated cytotoxicity. However, there was a ~15% residual cytotoxicity observed after MHC-I blockade perhaps suggesting a limited but

detectable NK cell response after all. Several factors could explain this. First, NK cells in mammals discriminate between healthy and non-healthy cells through their activating and inhibitory receptors, to activate NK cell functions and prevent attacks to healthy cells, respectively (Pegram et al., 2011). Inhibitory receptors recognize primarily classical and non-classical MHC-I molecules expressed on target cells. When a cell becomes infected, it may downregulate MHC-I molecules, generating a “missing self” signal that is recognized by these inhibitory receptors triggering the activation of NK cells (He & Tian, 2017; S. Kumar, 2018b). In this chapter, while fin cell MHC-I molecules were targeted with blocking antibodies, this was likely incomplete. Moreover, the antibody used was specific to classical MHC-I alleles, potentially leaving non-classical MHC-I molecules unblocked. If rainbow trout NK cells interact preferentially with non-classical MHC-I molecules, as in mammals, then these unblocked molecules may have continued inhibitory signals, limiting NK cell activation. Second, the splenocyte population used as effector cells may have limited functional NK-like cells or activating signals during co-culture. In mammals, NK cells not only depend on the absence of MHC-I molecules but also on cytokine expression and other activating signals (Kumar. S, 2018; Moretta & Moretta, 2004). Lastly, while NK-like cells have been reported in fish, the identity, activation signals, and receptors in rainbow trout are poorly understood (Fischer et al., 2013). It is possible that these cells respond differently to MHC-I modulation and require different activation conditions that were absent in this *in vitro* assay.

Interestingly, MHC-I blocking did not affect the expression of intracellular protein levels of the ER chaperones β 2m and tapasin indicating that the protein synthesis, folding and/or antigen loading of MHC-I molecules within the endoplasmic reticulum was not disrupted during MHC-I blockade experiments (Everett & Edidin, 2007; Garbi et al., 2000; Li et al., 2016). It could be that

there was a modest activation of the antigen presentation chaperones due to low or absent expression of pattern recognition receptors (PRRs) such as TLRs or RIGs which recognize viral RNA (Lester & Li, 2014; Rehwinkel & Gack, 2020) and activate the signaling cascade necessary for full activation of the antigen presentation pathway (Castro et al., 2014; Duan et al., 2022; Such et al., 2020). In addition, the dose and duration of viral antigen exposure could affect the intensity of the response, or the control cells have a high basal level of these proteins minimizing the changes in expression. Therefore, the reduced cytotoxic activity observed can be attributed to a disruption in the process of antigen recognition by CD8⁺ T cells, due to the antibody binding to MHC-I molecules resulting in the prevention of T cell receptor and CD8 coreceptor engagement, more than inhibition/activation of the antigen presentation chaperones which were activated under these experimental conditions.

In addition, the secretion of IFN γ and IL-2 was evaluated to assess T cell activation following antigen presentation by fin cells, as both cytokines are key during cytotoxic immunity in mammals and are considered crucial during CD8⁺ T cell activation (Sad et al., 1995). IFN γ promotes cell-mediated cytotoxicity (Bhat et al., 2017; Tau et al., 2001), while IL-2 increase T cell proliferation and survival (Cheng et al., 2002). In teleost fish, homologues of these cytokines have been described and have shown key functions related to fish immune responses (Díaz-Rosales et al., 2009; Hu et al., 2021; Wang et al., 2018; Zou et al., 2005); however, few detection and sensitive tools are available to quantify their expression at the protein level. The absence of detectable cytokine levels in the present assay may be due to suboptimal T cell activation, limited assay sensitivity or delayed secretion of these cytokines. Similarly, perforin a key effector molecule released by CD8⁺ T cells to lyse infected cells, was evaluated to determine cytotoxic pathways

involved in this *in vitro* assay. The lack of molecular tools to understand and study non-model organisms like rainbow trout, force researchers to rely on the use of non-specific tools, hoping for sufficient cross-reactivity between species. In this chapter, a rabbit anti-human perforin antibody was used to analyze the levels of perforin in VHSV-exposed fin cells and activated splenocytes after co-culture. However, no signal was detected, likely due to poor cross-reactivity with trout perforin. These results shows the technical barriers encountered when studying fish immunity and highlights the urgency for species-specific immunological reagents.

As mentioned, MHC-I-mediated recognition was crucial for the cytotoxic response from activated splenocytes against VHSV-exposed dorsal fin cells, but the question now is how exogenous viral antigens were processed and presented via MHC-I in our assay? It is important to remember that in this study heat-killed VHSV was used to activate splenocytes against this virus, and that the same preparation was used to stimulate dorsal fin cells for viral presentation onto MHC-I molecules (Figure 4.1). Since these inactivated viral particles cannot replicate and produce cytosolic proteins, the presentation on MHC-I likely occurred by other molecular pathways (Elveborg et al., 2022; Reynisdóttir et al., 1990). Under the endogenous antigen presentation pathway, cytosolic proteins are processed and presented on MHC-I molecules (Pishesha et al., 2022). However, in mammals, some specialized APCs, can internalize and process exogenous antigens and load them onto MHC-I molecules by a process called cross-presentation (Embgenbroich & Burgdorf, 2018). During this process extracellular antigens are taken up mainly by dendritic cells and macrophages and processed by proteasome or vacuole-dependent pathways to finally be loaded onto MHC-I molecules to be recognized by CD8⁺ T cells (Flinsenberget al., 2011; Kurts et al., 2010). Although several studies have shown the presence of macrophage-like

and dendritic-like cells, this pathway is not well characterized in teleost fish (Bassity & Clark, 2012; Hodgkinson et al., 2015; Lugo-Villarino et al., 2010). The fact that dorsal fin cells stimulated with heat-inactivated VHSV were able to activate a cytotoxic response suggest that they may have cross-presentation capacities (Bender et al., 1995; Lizée et al., 2003; Shi et al., 1998). In addition, since the cytotoxic response from activated splenocytes was in part MHC-I-dependent, it is suggested that the viral antigens from heat-inactivated VHSV were successfully processed and presented onto MHC-I molecules despite their extracellular origin (Embgenbroich & Burgdorf, 2018). On the other hand, it is important to notice the activation of splenocytes by heat-inactivated VHSV following intraperitoneal injection. Since the virus was inactivated by heat, the most probable route to reach immune organs like the spleen was through passive and active transportation via blood vessels (Kita & Itazawa, 1990; Wu et al., 2022). In fish, the spleen is considered a major immune tissue with similar functions, such as antigen trapping and T cell activation, as the mammalian counterpart (Ali et al., 2014; Deng et al., 2022; Kumar et al., 2018; Zapata, 2024), and it contains abundant in macrophages and dendritic-like cells which can potentially internalize and process viral antigens for presentation to resident T cells (Bassity & Clark, 2012; Iliev et al., 2013; Sun et al., 2024). In addition, in the peritoneum, immune cells like macrophages, dendritic cells and B cells may take up viral antigens at the site of injection and migrate to the spleen where the adaptive immune response could be activated by the antigen presentation pathway (Flessner & Dedrick, 1998; He et al., 2024). Although heat-inactivated VHSV lacks replication capacities, their structural proteins are still preserved, which make them immunogenic, and able to stimulate cellular responses after being processed by the fish immune system (Dubensky et al., 2012; Elveborg et al., 2022).

Once the antigen presentation assay using rainbow trout dorsal fin cells was standardized, the final goal of this chapter was to evaluate how suboptimal temperatures affect the recognition of viral antigens and activation of CD8⁺ T cells. Dorsal fin cells previously exposed to heat-inactivated VHSV IVb, were co-cultured with activated splenocytes at three temperatures: 10°C, 16°C and 22°C. As mentioned in previous sections, the optimal temperature for dorsal fin cell growth and normal behavior is 16°C, so any temperature below/above is considered suboptimal. Post co-culture at different temperatures, the cytotoxicity was analyzed by LDH release, which revealed a clear temperature-dependent pattern, in where cytotoxicity was significantly reduced at 10°C compared to higher temperatures. These findings validate the molecular and cellular observations made in previous chapters where the antigen processing and presentation was impaired at low temperatures. However, it is important to highlight that in this experiment, cells were exposed to different temperatures during the co-culture, that is, during the interaction between dorsal fin cells MHC-I and splenocytes TCR/CD8. Thus, the reduced cytotoxic response at lower temperatures was more likely to be an impairment in recognition rather than antigen processing. Even though the splenocytes were sensitized and pre-activated with VHSV IVb, their ability to recognize and kill previously VHSV-exposed fin cells was compromised due to low temperature exposure. This result suggests that stable MHC-I-TCR/CD8 recognition and membrane fluidity are a temperature-sensitive process in rainbow trout cells. It has been proven that low temperatures reduce cell membranes fluidity due to changes in the composition of phospholipids and fatty acids that directly affects membrane-bound molecules such as MHC-I and T cell receptors (Los & Murata, 2004), changing their mobility and clustering which is crucial during antigen recognition (Fooksman et al., 2006; Mocsár et al., 2016). Thus, while processing

of antigens by dorsal fin cells may be functioning, the final step of antigen recognition fails under lower temperatures.

In summary, this chapter established and validated a primary cell-based antigen presentation assay using rainbow trout dorsal fin cells. Flow cytometry confirmed that these cells express key antigen presentation markers, supporting their potential as antigen presenting cells. In addition, the assay was standardized to measure CD8⁺ T cell-mediated cytotoxicity using co-cultures with sensitized splenocytes and it was proved that MHC-I blocking significantly reduced cytotoxicity, confirming the contribution of MHC-I and CD8⁺ T cells during this response. Importantly, this recognition was temperature-sensitive, with low temperatures impairing the cytotoxic response from activated splenocytes against VHSV IVb. Together, these results provide a novel *in vitro* system to study and understand the antigen presentation pathway in fish species like rainbow trout. This is particularly relevant for aquaculture, where thermal stress is known to compromise immune function that could contribute to disease outbreaks. In addition, vaccine effectiveness is affected by temperature fluctuations, likely due to impaired immune system functions such as antigen presentation and T cell activation. Therefore, this assay not only offers a tool to better understand antigen presentation in fish, but also it is important to elucidate temperature-dependent immune function and vaccine development.

Chapter 5: General conclusions and future directions

Antigen presentation is a key process during the activation of adaptive immunity, allowing cytotoxic T cells to recognize and eliminate virus-infected host cells (Croft et al., 2019). While this process is well characterized in mammals, its molecular mechanisms in rainbow trout are not fully understood, especially during common environmental stressors like temperature fluctuations. With climate change increasing the frequency and severity of temperature shifts, which especially affects the water temperature of small water bodies, it is critical to understand how the immune system of ectotherms respond to these changes, especially during disease outbreaks provoked by pathogens such as the viral hemorrhagic septicemia virus (VHSV). This thesis investigated how temperature affects MHC-I complex formation, antigen processing, trafficking and CD8⁺ T cell activation in rainbow trout during an infection with VHSV. Results demonstrated that temperature directly impacts key steps of the antigen presentation and recognition of viral antigens.

Chapter 2 combined computational predictions, simulations and modeling with *in vitro* assays to elucidate the folding and assembly mechanisms of the rainbow trout MHC-I/β2m complexed with VHSV peptides at different temperatures. Bioinformatic tools such as NetChop 3.1, NetCTL 1.2, HADDOCK 2.4 and AlphaFold2, enabled the prediction and modeling of rainbow trout MHC-I molecules bound to its light chain β2m and complexed with VHSV-peptide candidates despite the absence of rainbow trout MHC-I crystal structures.

To evaluate the effects of temperature on complex stability, computational simulations using the webserver CABSflex 2.0 were performed to model structural flexibility across a range of temperature-like factors corresponding to low, moderate and high temperatures (0.5, 1.5 and 2.5, respectively). These simulations resulted in an increased flexibility with rising temperatures, particularly in loop regions, the α-helices forming the peptide binding groove and the α3 domain,

which is known to bind $\beta 2m$. Lower fluctuations with the glycoprotein peptide TVTFCGAEW were observed compared to the other VHSV-predicted peptides, which aligns with greater molecular docking results, reinforcing that peptide quality can influence MHC-I conformational stability affecting binding affinity. Interestingly, peptide C-terminal regions (residues 6-9) were especially affected by temperature suggesting a higher sensitivity in the anchoring residues under thermal stress.

A major achievement of this chapter was the successful expression and purification of rainbow trout MHC-I heavy chain and $\beta 2$ -microglobulin ($\beta 2m$), which is rare in the field, with only one other report in carp (Chen et al., 2017).. Although refolding efficiency was low, likely due to absence of other chaperones or low affinity of selected peptides, rainbow trout MHC-I/ $\beta 2m$ /VHSV-peptide complexes were obtained using the top peptide candidates, validating the predictive bioinformatic results. Alternative refolding strategies could be explored to improve folding efficiency in the future. Taken together, this chapter provides a better understanding of peptide–MHC-I interactions in teleosts and highlights key experimental and computational strategies to support vaccine design and disease resistance.

Having predicted VHSV peptides and tested their ability to bind and be folded with MHC-I complexes under different temperatures, the next step was to elucidate how temperature fluctuations affect other steps of this pathway. In Chapter 3, the effect of temperature on the antigen processing, trafficking and presentation was assessed by analyzing proteasome activity, MHC-I/ $\beta 2m$ surface expression, $\beta 2m$ release and IFN-I secretion in a controlled *in vitro* system. VHSV-infected rainbow trout cell lines (RTGill-W1, RTS11 and RTGut-GC) presented significantly reduced expression of MHC-I/ $\beta 2m$ complexes on the cell surface, which is a common evasion

mechanism used by viruses (Hewitt, 2003). This was particularly evident after exposure to moderate suboptimal temperatures (14°C), compared to other temperatures (4°C and 20°C).. This decrease in MHC-I/β2m complexes was correlated with an increase in the release of β2m to the extracellular space which is common when unstable complexes are expressed on the cell surface (Montealegre et al., 2015). These results suggest a disruption of MHC-I trafficking and/or the production of sub-optimally loaded complexes under moderate cold stress during an infection with VHSV. The latter was not observed under low temperatures (4°C), proving that temperature alone does not affect the host immune system, but pathogen behavior is also a factor, since both control and infected groups showed no differences. While the trafficking and presentation was impaired during thermal stress, apparently the processing and antiviral responses were not affected since high proteasomal activity was observed together with high secretion of the antiviral cytokine IFN-I. These results provide molecular evidence on how temperature affects the antigen presentation pathway in rainbow trout, which is a crucial process during antiviral responses. In addition, this data offers a cellular explanation for VHSV outbreaks especially during seasons where the temperature drops drastically.

This thermal sensitivity after VHSV infections was shown to have consequences to activate adaptive immunity using an *in vivo/ex vivo* approach. In Chapter 4, an antigen presentation assay was developed and validated using primary dorsal fin cells co-cultured splenocytes from VHSV-sensitized rainbow trout. The novel use of fin cells as antigen presenting cells (APCs), provided an *in vitro* assay in where TCR/MHC-I are genetically identical especially important when isogenic rainbow trout cell lines and/or animals are not available. Flow cytometry analysis confirmed that fin cells express both MHC-I and MHC-IIα, together with low expression of CD3ε

and IgM, suggesting a heterogeneous population with immune functions. Importantly, a cytotoxic response from VHSV-activated splenocytes against fin cells exposed to heat killed VHSV antigens was observed, suggesting not only an antigen specific recognition but also the capacity of rainbow trout fin cells for cross-presentation; a process that has not been characterized in fish. This key observation is particularly relevant not only at a basic science level but also for vaccine development, proving that inactivated viral vaccines may still activate cellular immunity through cross-presentation pathways.

In addition, the blockade of MHC-I molecules on fin cells by using an anti-MHC-I antibody reduced the cytotoxic activity from activated splenocytes by 50%, suggesting a strong MHC-I contribution during this killing process, probably performed by CD8⁺ T cells. While a compensatory activation of NK cells due was expected to the absence of MHC-I molecules on the cell surface, this was not observed. In fish, the characterization of functional NK-like cells remains poorly understood. These results suggest that NK-like cell activation requires different co-stimulatory signals, or they were absent during this assay. In addition, MHC-I blocking did not affected the intracellular expression of β 2m and tapasin, suggesting that the interference was only during antigen recognition and not during antigen processing.

Once the antigen presentation assay was validated, the effect of suboptimal temperatures on antigen recognition and T cell activation was evaluated. Activated splenocytes and VHSV-exposed fin cells were co-cultured at different temperatures, and the cytotoxic response was analyzed. Interestingly, low temperatures (10°C) presented lower cytotoxic activity compared to optimal (16°C) and high (22°C) temperatures, supporting previous results in where low temperatures impaired antigen processing and presentation. These results not only provide, a novel

antigen presentation assay *in vitro*, using rainbow trout fin cells, but also created an advance in the knowledge of the antigen presentation pathway in rainbow and how suboptimal temperatures can affect this process during viral infections.

Future research should follow several directions to continue the characterization of the antigen presentation pathway in rainbow trout under environmental stressors and infections. One important next step is to perform immunopeptidome analysis to identify naturally produced peptides bound to MHC-I molecules in rainbow trout during an infection with VHSV under different thermal conditions. This will validate the bioinformatic results and will help to elucidate how thermal stress affects peptide repertoires. Having these naturally produced peptides, together with a large-scale protein purification of MHC-I molecules and related chaperones, will help to perform DSF assays and resolve crystal structures of the antigen presentation pathway in rainbow trout for the first time. This would significantly advance the understanding of the structure and function of fish MHC-I molecules and its interacting partners, contributing to the current knowledge gap in antigen presentation in teleost fish.

In addition, live-cell imaging of MHC-I trafficking and recycling under thermal stress during an infection with VHSV, will help to localize the exact disruption in intracellular transport. Beside this, the production and purification of antibodies against key chaperones of the antigen presentation pathway, will help to identify their function during MHC-I molecules trafficking and recycling. This could be combined by using different inhibitors to identify specific steps disrupted during antigen processing and presentation under thermal stress during a viral infection. For example, inhibitors of proteasomes, immunoproteasomes, TAP transporter, Golgi apparatus and lysosomes, would be helpful to address this.

A critical next step using the fin-cell based antigen presentation assay would be the evaluation of the cellular and molecular mechanisms of CD8⁺ T cell-mediated cytotoxicity in rainbow trout and other species. The use of an anti-CD8 antibody would directly confirm the contribution of these cells during the cytotoxic response observed in this thesis. Additionally, characterizing the cytotoxic mechanisms such as the expression of perforin, granzyme and Fas/FasL system, would show the exact killing mechanism used by these cells, and how is affected under thermal stress. While cytokine secretion was measured in this study, limited antibody sensitivity resulted in no signal detection; therefore, more sensitive detection tools are necessary to fully investigate the cytokine profile during the activation of T cells. In addition to cytokine secretion, T cell proliferation is a key step during cell-mediated cytotoxicity proving activation, so this would be a valuable data to have offering a deep insight into the strength and duration of the cytotoxic response under different temperatures. Finally, since the use of heat-killed VHSV elicited a cytotoxic response from splenocytes, long-term studies could explore whether repeated immunizations and antigen persistence can activate durable CD8⁺ T cell memory which has not been proved in teleost fish.

Altogether, this work offers an advance in understanding a poorly characterized immune process that is crucial during viral infections. These results demonstrated that suboptimal temperatures disrupt multiple steps of the antigen presentation pathway in rainbow trout during an infection with VHSV, from MHC-I complex stability to cell surface expression and CD8⁺ T cell recognition and activation. With the combination of computational simulations and modelling, *in vitro* assays, and a novel antigen presentation assay, this thesis provide a comprehensive understanding of the functionality of the antigen presentation pathway and the effects of

temperature in rainbow trout. Importantly, this approach can be used to study other pathogens, particularly those that exploit thermal stress to increase infectivity, and can also be adapted to other species.

On the other hand, these findings offer a molecular explanation for disease susceptibility during thermal stress by showing that suboptimal temperatures reduces the stability, surface expression of MHC-I/ β 2M complexes and the activation of CD8⁺ T cell responses. This information can directly improve vaccine scheduling in aquaculture; for example, avoiding vaccination during cold seasons or during seasons where cold snaps or heat waves are more prominent, since the immune response is likely to be weaker. Instead, vaccination should be implemented when immune functions such as MHC-I antigen presentation is more efficient. Moreover, the antigen presentation assay developed using dorsal fin cells can be used as a pre-screening tool for hatcheries and vaccine producers, to evaluate immunogenicity of multiple viral peptides before starting with costly *in vivo* trials. At the same time, MHC-I stabilizing adjuvants with existing vaccines can be applied to maintain and improve fish health.

Additionally, the successful expression, refolding and modeling of the rainbow trout MHC-I and β 2m proteins offers a molecular tool to design peptide-based vaccines by testing the MHC-I binding affinity. These tools could be used to identify peptides that form thermally stable complexes to formulate vaccines that are more effective across temperature ranges commonly experience in pond or net-pen systems, maintaining the immunogenicity of the vaccine during both summer heat waves and cold winters. Furthermore, the *in vitro* methods presented here could be adapted to other aquatic species and pathogens, especially the ones that are more infective during thermal stress.

In summary, in the context of climate change, where shallow ponds or open net-pens are increasingly exposed to rapid temperature fluctuations, this work not only fills a key knowledge gap in antigen presentation pathway in rainbow trout but also established the link between environmental temperature and antigen presentation efficiency. This thesis provides molecular and cellular tools supporting the development of temperature-adapted tools and immunization strategies to improve vaccine development, immune monitoring and disease prevention protocols in aquaculture.

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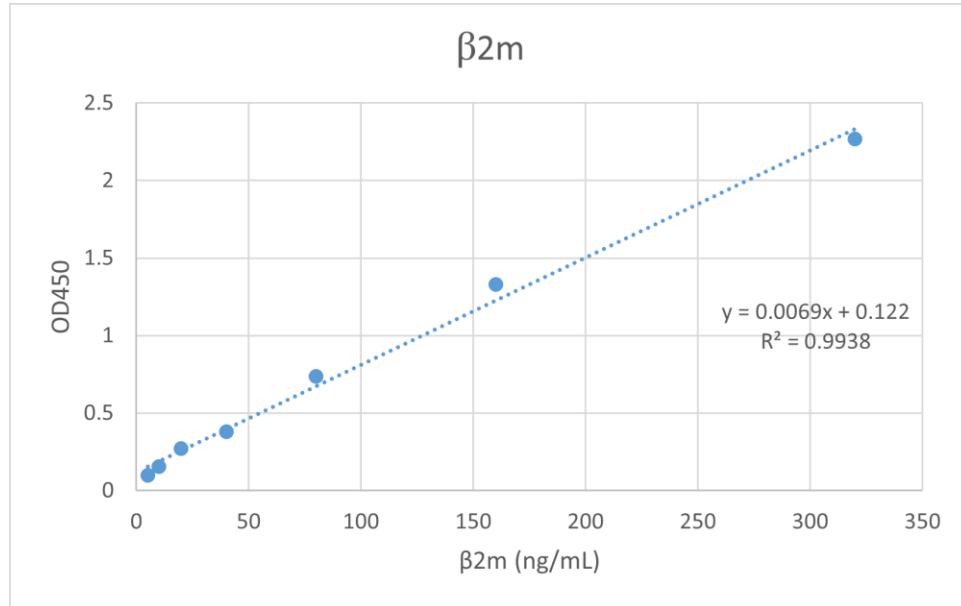
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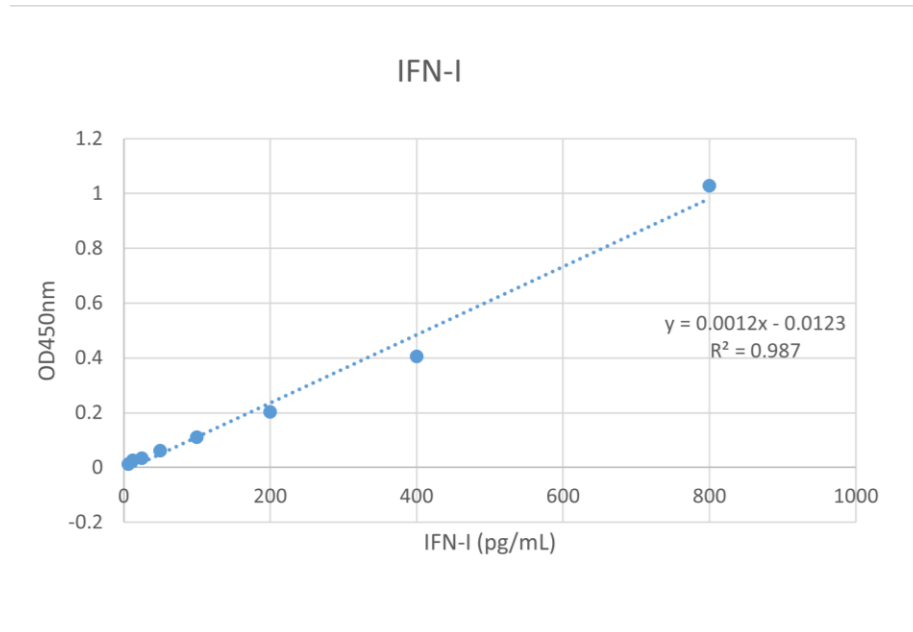
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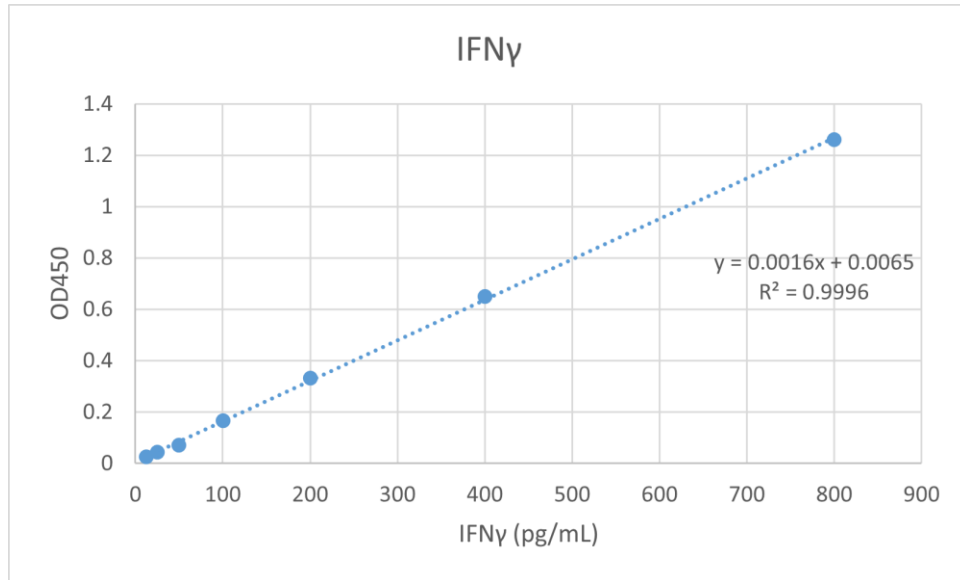
Appendix A: Supplementary Figures



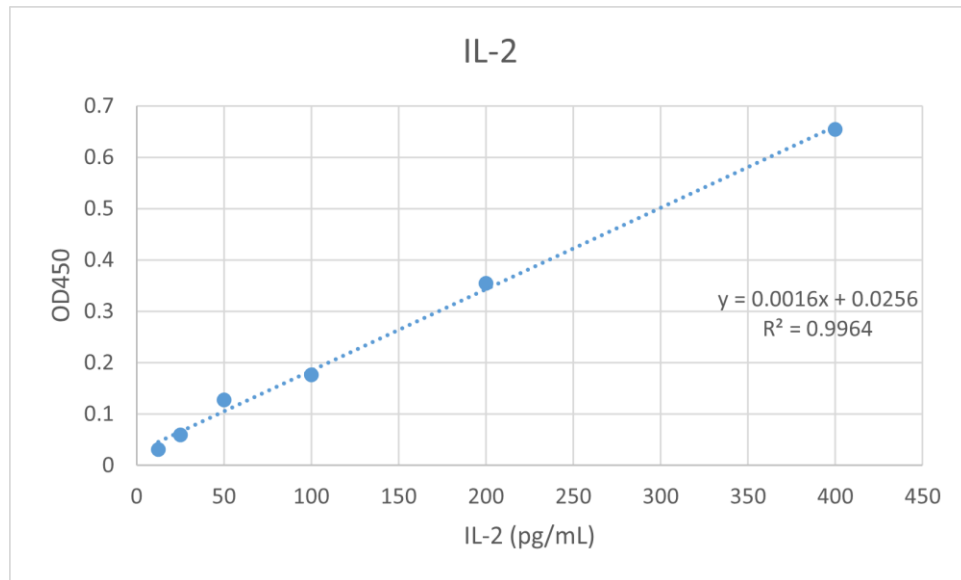
Supplementary Figure S.1. Standard curve of recombinant rainbow trout $\beta 2m$ using indirect ELISA. A two-fold serial dilution of recombinant rainbow trout $\beta 2m$, ranging from 320 ng/mL to 5 ng/mL was prepared in L-15 medium containing 2% FBS for use in an indirect ELISA. Each concentration was tested in triplicate. Mean absorbance values were obtained and subtracted from the blank. A linear regression analysis was performed, and the resulting equation along with the R^2 value is shown.



Supplementary Figure S.2. Standard curve of recombinant rainbow trout IFN-I on sandwich ELISA. A two-fold serial dilution of recombinant rainbow trout IFN-I, ranging from 800 pg/mL to 6.25 pg/mL was prepared in L-15 medium containing 2% FBS for use in a sandwich ELISA. Each concentration was tested in triplicate. Mean absorbance values were obtained and subtracted from the blank. A linear regression analysis was performed, and the resulting equation along with the R^2 value is shown.



Supplementary Figure S.3. Standard curve of recombinant rainbow trout IFN γ on sandwich ELISA. A two-fold serial dilution of recombinant rainbow trout IFN γ , ranging from 800 pg/mL to 12.5 pg/mL was prepared in L-15 medium containing 2% FBS for use in a sandwich ELISA. Each concentration was tested in triplicate. Mean absorbance values were obtained and subtracted from the blank. A linear regression analysis was performed, and the resulting equation along with the R^2 value is shown.



Supplementary Figure S.4. Standard curve of recombinant rainbow trout IL-2 on sandwich ELISA. A two-fold serial dilution of recombinant rainbow trout IL-2, ranging from 400 pg/mL to 12.5 pg/mL was prepared in L-15 medium containing 2% FBS for use in a sandwich ELISA. Each concentration was tested in triplicate. Mean absorbance values were obtained and subtracted from the blank. A linear regression analysis was performed, and the resulting equation along with the R^2 value is shown.