Cave ille qui didicit ambulare extra infernum

# THE PHYSIOLOGICAL COST OF A SUB-LETHAL INFECTION WITH INFECTIOUS SALMON ANEMIA VIRUS (ISAV) IN WILD ATLANTIC SALMON (*SALMO SALAR*)

by

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# ABSTRACT

Infectious Salmon Anemia Virus (ISAV) is an important pathogen affecting farmed Atlantic salmon and infectious to wild salmon. Wild Atlantic salmon in the outer Bay of Fundy of Canada's East Coast is an important component of biodiversity in aquatic ecosystems and has been endangered since 2010. Here, the effects of sub-lethal ISAV infection are quantified in wild-type Atlantic salmon (Tobique River strain) using an ISAV strain first isolated in Nova Scotia, Canada, in 2012. All fish were intraperitoneally injected with the virus or sham-injected with physiological saline, and anemia caused by ISAV was assessed at intervals using red blood cell count, hematocrit, and hemoglobin concentration. The effects of ISAV infection on metabolic rates at peak infection (16 days post-infection; DPI) and post-peak infection (30 DPI) were measured using whole-tank intermittent stop-flow respirometry to obtain post-stress aerobic metabolic scope (PSAMS) and excess post-stress oxygen consumption rates (EXPOC) using a 2-minute net chase as the stressor. No anemia was observed at 16 or 30 DPI. ISAV had no measurable effect on the fish during this experiment. In a separate experiment, ISAV challenged fish kidney tissue was sampled at 7, 17, and 78 DPI, with respective relative viral loads of $10^4$ RNA copies, $10^5$ RNA copies, and $10^{3.08}$ RNA copies. The effects of sub-lethal ISAV infection on the protein synthesis/degradation cycle was measured. Anemia was observed at 7 and 17 DPI in ISAV infected fish for this experiment, and protein synthesis rates were higher in ISAV+ fish compared to sham-injected controls at 7 DPI (7.51 ± 1.75 %/day; $p = 0.001$) and 17 DPI (7.65 ± 1.73 %/day; $p = 0.06$).

DEDICATION

Cette thèse est dédiée à mes parents.

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# LIST OF SYMBOLS, NOMENCLATURE OR ABBREVIATIONS

AMS: Aerobic Metabolic Scope

ANOVA: Analysis of Variance

ASK: Atlantic Salmon Kidney Cell Line

BMR: Basal Metabolic Rate

CAD: Canadian Dollar

CCAC: Canadian Council on Animal Care

CFIA: Canadian Food Inspection Agency

COSEWIC: Committee on the Status of Endangered Wildlife in Canada

D5-PHE: ring-D5-Phenylalanine

DFO: Department of Fisheries and Oceans

DFO-GFC-MBU: Department of Fisheries and Oceans, Gulf Fisheries Centre, Molecular Biology Unit

DPI: Days Post-Injection

EPOC: Excess Post-Exercise Oxygen Consumption

EXPOC: Excess Post-Stress Oxygen Consumption

F: Fusion Protein

GC-MS: Gas Chromatography with Mass Spectrometry detection

GFC: Gulf Fisheries Centre

Hb: Hemoglobin

HBSS: Hank's Buffered Salt Solution

HE: Hemagglutinin-esterase

HPR: Highly Polymorphic Region

HPR0: Strain of ISAV with full length HPR

HPRΔ: Strain of ISAV with a deletion in the HPR segment. May also be followed by a number e.g., HPR4

IP: Intraperitoneal

ISAV: Infectious Salmon Anemia Virus

ISAV+: ISAV Positive

Ks: Fractional Rate of Protein Synthesis

MBU: Molecular Biology Unit

MID: Minimal Infectious Dose

$MO_2$: Mass-specific oxygen consumption rate

MR: Metabolic Rate

MS222: Tricaine Methanesulfonate

NB: New Brunswick

NS/2012: Strain of ISAV from Nova Scotia, isolated in 2012, case 2012-21 (GFC full name is ISAV NS/2012-21 H17:11 NA)

OIE: Office International des Epizooties

PCA: Perchloric Acid

PFBBr: Pentafluorobenzyl Bromide

PSAMS: Post-Stress Aerobic Metabolic Scope

RBCC: Red Blood Cell Count

RMR: Routine Metabolic Rate

RT-qPCR: Real Time Quantitative Polymerase Chain Reaction

SABS: St. Andrews Biological Station

SHHb: Standard Human Hemoglobin

SMR: Standard Metabolic Rate

SPE: Solid Phase Extraction

USD: United States of America Dollar

UV: Ultraviolet

# CHAPTER 1- INTRODUCTION

## 1.1 Wild Atlantic salmon populations

Wild Atlantic salmon (*Salmo salar*) have historically been, and continue to be, prized and highly valued in New Brunswick (NB), Canada, as economic drivers of ecotourism (Pinfold 2011). Wild salmon are important for aquatic ecosystem biodiversity and resilience, as they are vectors for transferring marine nutrients to river ecosystems (Naiman *et al*. 2002, McLennan *et al*. 2019). Wild salmon are also significant to indigenous peoples as food and festive cultural celebrations (Ween and Colombi 2013).

From 1970 to 2014, there was a decline of 60-70% in wild Atlantic salmon on both sides of the Atlantic Ocean (Friedland *et al.* 2014). High-seas fishing catches from the Faroe Islands, Norway and Greenland declined by 1000 tonnes/year from 1968 to 1991 (Dadswell *et al.* 2010), and these fisheries were closed in 1991, 1999, and 2002, respectively. In Canada, a decline in high-seas capture of Atlantic salmon was first observed in the 1970s (DFO 2019). Fisheries management measures were implemented by the Department of Fisheries and Oceans Canada (DFO) in 1984, leading to a complete moratorium on Atlantic salmon commercial fisheries in 2000 (DFO 2020). Canadian commercial Atlantic salmon fisheries remain closed while the Indigenous provisional harvests (54.0 tonnes) and recreational fisheries (38.1 tonnes) were dincreased in 2019 (ICES 2020).

As wild salmon populations continue to decline (Parish *et al*. 1998, Wilke *et al.* 2015, Soto *et al.* 2018), the conservation of this species is essential for stock rehabilitation (McConnell *et al.* 1997) and maintain healthy biodiversity for local ecosystems. In May

2001, wild Atlantic salmon populations in NB's inner Bay of Fundy area were listed as endangered by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC); this was reconfirmed in 2006. In November 2010, wild Atlantic salmon populations in NB's outer Bay of Fundy area were listed as endangered by the COSEWIC (DFO 2014, DFO 2021). Efforts to improve wild salmon conservation are important and as such, the smolt to adult supplementation program at the Mactaquac DFO facility is an effort to maintain and build up the endangered population of salmon. Wild salmon caught as smolts from the Tobique River in NB and raised at the Mactaquac facility were spawned. These $F_1$ progeny are the fish that were used for this thesis' research.

### 1.2 Farmed Atlantic salmon

Aquaculture production, excluding aquatic macrophytes, has risen globally to 80 million metric tonnes, valued at $ 232 billion per annum (Garlock *et al.* 2020). Total net aquaculture production in Canada was valued at $487.4 million by the DFO in 2018, all species considered (DFO 2018). Farmed Atlantic salmon is a valuable contribution to these global and national standings.

Globally, the first commercial aquaculture for Atlantic salmon was started in 1970 and produced 10 tonnes of fish; 25 years later, the global yield was well over 400 000 tonnes; in New Brunswick, Canada, these numbers went from 6 tonnes to over 16 000 tonnes within that same timeframe (Carr *et al.* 1997). Globally, Atlantic salmon aquaculture has grown to produce 1 821 352 tonnes in 2020 (ICES 2021). The rapid growth of Atlantic salmon aquaculture can be attributed to many factors, such as the nutritional value of fish oils (omega-3 fatty acids) (Williams 1988), global protein

consumption, food security, employment income (Kobayashi *et al.* 2015, Belton *et al.* 2018), and the decline of wild fisheries.

Norway is the world's leading producer of farmed Atlantic salmon, with 1 393 108 tonnes in 2019 (ICES 2021). Canada sits at fourth place for global Atlantic salmon production with 118 630 metric tonnes nationally in 2019 (DFO 2021 b), including Atlantic and West Coast production. In 2019 in Canada, the Atlantic Provinces produced 36 174 tonnes of Atlantic salmon, making it the third-largest producer in the Northern Atlantic region (ICES 2021).

New Brunswick's salmon farming harvest began in 1978 with one site and has since expanded to over 90 sites that are responsible for 40% of Canadian aquaculture production (Ford and Myers 2008, Gov of New Brunswick 2017) with 28 289 tonnes produced in 2018 and 22 395 tonnes produced in 2019 (Gov of New Brunswick 2018, Gov of New Brunswick 2019 a). Salmon export values for NB were valued at CAD 313.9 million in 2019, still second to the lobster export industry valued at CAD 962.1 million (Gov of New Brunswick 2019 b).

### 1.3 Interactions between wild and farmed fish and pathogen transfer

Atlantic salmon aquaculture has the highest proportion of fish-derived breeding programs globally in aquaculture (Teletchea and Fountain 2014). Some of these populations have experienced upwards of 12 generations of domestication and are genetically distinct from their wild counterparts to express various traits (Glover *et al*. 2017). As aquaculture production continues to grow worldwide (ICES 2020), so does the number of farmed Atlantic salmon, vastly outnumbering local wild populations.

Aquaculture sites can have farmed salmon population densities between 22 and 25 kg/$m^3$ per cage (Turnbull *et al*. 2005, Liu *et al.* 2017). This growth in population size of domesticated salmon and aquaculture site abundance augments the number of farmed fish escapes into the wild. Farm escapees into the Northern Atlantic Ocean were estimated at 2 million individuals per year in 2003 (Schiermeier 2003). An average of 4.3% of Norwegian farmed salmon escapes to the wild annually, in numbers varying from 38 000 (2012) to 921 000 (2006) (Svenning *et al.* 2017). Interactions with escapees have been linked to declining wild salmon populations in proximity to aquaculture sites and associated risks of disease transmission between these populations (Castellini *et al.* 2020, Ford and Myers 2008, Finstad *et al.* 2011, Svenning *et al.* 2017).

The transfer of infectious pathogens between farmed and wild salmon populations is not unidirectional. Spillover refers to pathogen transmission from wild to farmed fish, and spillback is the transmission of farmed fish pathogens to wild populations (Daszak *et al.* 2000, Krkošek 2017). In most cases, sympatric wild salmon transfer pathogens to coastal salmon farm sites (Kurath and Winton 2011, Miller *et al*. 2014) as migration routes to and from the ocean pass through or within proximity of cage sites (Nowak 2007, Ford and Myers 2008, Walker and Winton 2010). Once a pathogen enters the aquaculture site, it can move rapidly through the population by serial passage among hosts (Ebert 1998). In a natural environment, one possible evolutionary path for pathogens with initially high virulence levels is to gradually decrease virulence over time with each new series of host passages, leading to an eventual equilibrium of optimal virulence and a commensalism type of pathogen-host relationship (Griffiths 1999, Bull and Lauring 2014, Vu and Kaiser 2017).

Numerous environmental factors contribute to a pathogen's level of virulence (Cressler *et al.* 2016). Aquaculture sites have been observed to support the artificial selection of higher virulence in pathogens. Kennedy *et al.* (2015) identified eight standard aquaculture practices that affect the evolution of virulence: rearing at high densities, compression of the rearing cycle, use of broodstock with limited genetic diversity, transfer of endemic disease in cultured populations, vaccination, breeding for disease resistance, chemotherapy and reducing vertical transmission of pathogens. It has been proposed that low-level virulent strains can attain higher virulence levels, making them specialists in that specific farm environment (Nylund *et al.* 2019, Cárdenas *et al*. 2017). This change in virulence can lead to the creation of a reservoir for pathogens with potentially higher virulence levels within the aquaculture site.

### 1.4 Infectious Salmon Anemia Virus (ISAV) classification and structure

ISAV is the etiological agent causing Infectious Salmon Anemia (ISA) and is the sole member of the *Isavirus* genus within the Orthomyxoviridae family (Falk *et al.* 1997, Ritchie *et al*. 2009, Cardenas *et al.* 2017). ISAV shares similar characteristics with other family members, notably the influenza viruses (Dannevig *et al.* 1995, Clouthier *et al.* 2002). There are two major monophyletic clusters in the evolution of the ISAV: one European (strains from Norway, United Kingdom as well as Chile and the Faroe Islands) and the other North American (strains from Canada and the United States) (Krossøy *et al.* 2001, Rimstad and Markussen 2019). Within North America, a separate genotype of ISAV is genetically closer to the European ISAV, which indicates a more recent introduction of the virus (Gagné and Leblanc 2017).

Segment 6 of the viral genome contains a highly polymorphic region (HPR). Strains of ISAV with no deletion in their HPR are non-pathogenic and are not known to cause disease in salmon; these are referred to as HPR0. Viruses of this type are predominantly detected in gill epithelial cells in salmon (Christiansen *et al.* 2011, 2017). There is evidence that HPR0 is the ancestral form of the virus and that it subsequently evolved from a non-virulent form into the HPRΔ virulent forms (Christiansen *et al*. 2017). These have deletions in HPR and can cause systemic infections of endothelial and epithelial cells (Workenhe *et al.* 2007, Weli *et al.* 2013). All pathogenic strains of ISAV identified to date have a deletion in the HPR region (Christiansen *et al.* 2011, Mjaaland *et al*. 2002). HPRΔ strains can be cultured *in vitro* on cells, e.g., Atlantic Salmon Kidney (ASK) cells, whereas HPR0 does not replicate in cell culture.

ISAV is enveloped and composed of 8 single-stranded RNA segments of negative polarity (Mjaaland *et al.* 2002, Nylund *et al.* 2003), encoding at least ten known proteins (Falk *et al*. 1997 and Clouthier *et al.* 2002). Segment 8 of the genome codes for two open reading frames (ORF): the matrix protein (M) and s8ORF2 (Garcia-Rosado *et al*. 2008, Olsen *et al.* 2016). Atlantic salmon, as well as Atlantic salmon kidney (ASKASK) cell lines infected with ISAV produce and activate Type I interferons (Jensen and Robertsen 2002, Kileng *et al*. 2007, Leblanc *et al.* 2012).

The viral envelope surface has two important glycoproteins: hemagglutinin-esterase (HE) and a fusion protein (F), respectively encoded by genome segments 6 (Falk *et al*. 2004) and 5 (Aspehaug *et al*. 2005). The two main functions of HE are to promote viral attachment through a receptor-binding protein that interacts with sialic acid in host cells (Eliassen *et al.* 2000, Mikalson *et al*. 2005) and to allow the release of newly formed

virions via its acetyl esterase function within the host cell, a contributing factor to the level of virulence (Muller *et al.* 2010, Cook *et al.* 2017, Rimstad and Markussen 2019). The HE protein has one of the highest rates of mutation in the North American strains of ISAV at $1.13x10^{-3}$ nucleotides per site per year (Kibenge *et al*. 2007) as it is subjected to higher selective pressures from the host immune system. Olsen *et al.* (2012) observed no increase in viral load after ten serial passages of ISAV in farmed rainbow trout (*Oncorhynchus mykiss*) but did see nucleotide changes leading to one amino acid substitution. The effects of serial passage of ISAV have not been studied directly in wild salmon populations (La and Cook 2011), as weakened wild salmon fall prey to predation, making it harder to quantify effects (Miller *et al.* 2014).

Once cleaved, the F protein exposes the fusion peptide (Aspehaug *et al*. 2005, Cook *et al.* 2017) that attaches to host cell receptors allowing ISAV entry through the cellular membrane (Ojeda *et al.* 2020). Substitutions and insertions within the F gene on segment 5 have only been found in HPRΔ strains. A strain found in Chile (ISAV HPR 7b) initially had an insertion in the F protein segment; during this strain's resurgence, the mutation was no longer found. The virulence of this strain was lowered (Godoy *et al*. 2014, Rimstad and Markussen 2019), suggesting the importance of the HE and F proteins in determining a particular strain's virulence level. The progression to pathogenicity is hypothesized to be a two-step process with a deletion in the HPR0 HE gene (Cárdenas *et al*. 2014, Christiansen *et al*. 2017) and insertion or substitution in the F protein region. Other factors influencing pathogenicity include viral replication rate, host shedding rate, the level of host immune response, and the exposure level (Jones and MacKinnon 1999, Lauscher *et al.* 2011, Rimstad and Markussen 2019). There is evidence that resistance to

ISAV varies with the genetic background of farmed Atlantic salmon (Holborn et al. 2020, Gervais et al. 2021).

### 1.5 Symptoms of ISA

Clinical signs of ISA include swelling of the liver and kidney and ascites (accumulation of fluid in the peritoneal spaces) (Rimstad *et al*. 1999, Rimstad and Mjaaland 2002). ISAV is endotheliotropic, causing a generalized infection in the vascular system, and has been observed on salmon erythrocyte surfaces (Aamelfot *et al.* 2012). ISAV has also been observed to infect primary gill epithelial cells of host salmon (Weli *et al.* 2013). Head kidney leukocytes have been observed to be important target cells for ISAV (Danniveg *et al*. 1994, 1995). Red blood cells have also been observed to be hemophagocytosed (Aamelfot *et al.* 2014). In the final stages of severe infection, the virus causes severe anemia (hematocrit < 10%) and pathological changes consistent with a collapse of the circulatory system (Rimstad *et al.* 2011, Cook *et al*. 2017) that can result in death. Some fish recover from the infection, and some virus strains cause very low mortalities; the fish source can be a deciding factor on the level of virulence (Aamelfot *et al*. 2014, LeBlanc *et al*. 2018). Of all the North American strains examined to date, the most virulent is NA-HPR-4 (970-1) (Leblanc *et al.* 2010), also known as HPR-21 (Kibenge *et al*. 2007) but referred to as HPR-4 hereafter. *In vivo* studies using HPR-4 demonstrate a mortality rate greater than 90% within ten days of infection, a high infection rate, and the infection lasting ~ 40 days (Ritchie *et al*. 2009, Gagné and Leblanc 2017). ISA only develops in salmonid species. Atlantic salmon (*S.salar*) are the primary host; however, other members of the salmonid species, such as the amago trout

(*Oncorhynchus masou*), can be infected and develop ISA. Fish species outside the salmonids can be carriers of the virus without developing an infection; these include Atlantic cod (*Gadus morhua*), Atlantic herring (*Clupea harrengus*). However, these species (*G. morhua* and *C. harrengus*) have incomplete evidence for susceptibility (OIE 2016).

ISAV HPRΔ strains have a wide range of virulence. Daily sea-cage mortality can range from 0.05% to 0.1% of the population and, if left without treatment, can lead to cumulative mortality from 15% to 100% (Rimstad *et al.* 1999, Ritchie *et al*. 2009, Aamelfot *et al*. 2014, Gagné and Leblanc 2017). ISAV can cause severe losses in salmon farms. This virus almost destroyed the aquaculture industry on the Canadian East Coast when discovered during outbreaks starting in 1995 (Jones and Mackinnon 1999, Leblanc *et al.* 2016). Once a site was infected with ISAV, it was common practice to cull the whole population, disinfect and fallow the site, and start anew (OIE 2016). Qviller *et al.* (2020) observed that culling of ISAV-infected cages should be done as quickly as possible to avoid spreading the virus.

### 1.6 ISAV historical epidemiology, Office International des Epizooties (OIE) and Federal Reporting

The first officially registered outbreak of ISAV was in Norway in 1984 (Thorud and Djupvik 1988). It was acquired primarily by horizontal transmission from wild fish to farmed smolts and then from farm to farm (Nylund *et al*. 1994, Nylund *et al.* 2019). In 1990, ISAV was recognized by the "Office International des Epizooties" (OIE) (Plarre *et al.* 2011) and was listed as a reportable disease in 1995 (OIE 2006a, 2006b). A reportable

disease must be reported immediately to a veterinarian, as mentioned in the Health of Animals Act. If that veterinarian suspects it to be ISA, or any other listed reportable disease, a Canadian Food Inspection Agency (CFIA) veterinary inspector must follow up (CFIA 20a2020a). Canadian salmon aquaculture sites reported their first ISA cases in 1996 and ISAV was first isolated in NB in 1997 (Bouchard *et al*. 1999) with repeated outbreaks since then and it. It still remains a severe threat in Atlantic Canada (Mullins *et al.* 1998, Lovely *et al.* 1999, Plarre *et al.* 2011, Cook *et al*. 2017, Leblanc *et al*. 2018). If ISAV is detected in a country's animals or products, a compartment (e.g., a fish hatchery), or a zone, they cannot be exported (OIE 2016). Outbreaks of ISA were reported from 1997 to 2006, and more than 20 variants of HPRΔ ISAV have been observed in NB's Bay of Fundy (Johnson *et al*. 2008, Gagné and LeBlanc 2017). There were few reports of this virus from 2007 to 2011, although HPR0 was detected on farms (Leblanc *et al*. 2016). Since 2012, outbreaks have been reported regularly (CFIA 2020 a). There is strong evidence that these newer outbreaks stem from the maintenance of HPR0 and low-pathogenic HPRΔ strains in both farmed and wild salmon populations (Christiansen *et al*. 2011, Gagné and Leblanc 2017, Nylund *et al.* 2019).

### 1.7 ISAV in wild vs. farmed populations

ISAV has been detected in wild and farmed Atlantic salmon populations in marine and freshwater environments (Raynard *et al*. 2001, Plarre *et al.* 2005, Lyngstad *et al*. 2008).

Miller *et al.* (2014) outlined the current lack of research on the disease in wild salmon populations due to various limitations and complex interactions with many factors, such

as predation of diseased individuals. While ISA does not seem to be a leading factor in the decline of wild stocks (Nylund *et al.* 2003), there is little data available to estimate the effects of a change in virulence levels and the impact on wild populations (Nylund *et al*. 2019). In Norway, wild salmon populations only have ISAV-HPR0 infections (Madhun *et al*. 2019). Presently the farmed populations on Canada's East Coast are experiencing infections with various strains of ISAV of lower virulence than observed in historical epidemics, and ISAV infections have not been reported in wild salmonids. However, wild Atlantic salmon and brown trout (*Salmo trutta*) are thought to be carriers (Rimstad *et al.* 2011, LeBlanc *et al*. 2018, OIE 2016).

Once ISAV HPRΔ has reached critical prevalence and shedding rates, it can escape the site through spillback. Fish farm escapees can be carriers of ISAV. Shedding of mucus, urine, and fecal matter contributes to the spread of ISAV (Nylund *et al*. 1994, Totland *et al*. 1996). Salmon lice (*Lepeophtheirus salmonis* and *Caligus rogercresseyi*) can also be vectors of ISAV (Nylund *et al*. 1993 Oelckers *et al.* 2014, Barker *et al*. 2019) though not a primary contributing factor of infection.

### 1.8 Metabolic rate

Metabolic rate (MR) represents the energetic cost for an organism to function. Oxygen is a biological requirement for most animals, including salmon (Thorarensen and Farrell 2011). Aerobic metabolism relies on oxygen uptake from the air or water and can be measured as the mass of oxygen consumed per unit of time, termed oxygen consumption rate ($MO_2$) (Nelson 2016, Chabot *et al.* 2016). The minimum oxygen consumption to support essential homeostatic activities of an endothermic organism is

commonly termed the basal metabolic rate (BMR); for ectothermic organisms, it is termed standard metabolic rate (SMR) (Chabot *et al.* 2016). Aerobic metabolic scope (AMS) is the difference between maximum metabolic rate (MMR) and SMR, giving the total amount of aerobic energy available for other physiological processes such as digestion, reproduction, locomotion, and growth (Rosewarne *et al.* 2016). MMR is typically measured when the fish is exercised to the point of exhaustion (Norin and Clark 2016). Here the post-stress aerobic metabolic scope (PSAMS; details provided in the Methods section) is used instead of AMS.

After a stressful event that influences the fish's oxygen consumption, there is a three-phase recovery back to the SMR, and known as the excess post-exercise oxygen consumption (EPOC) (Zhang *et al.* 2018). These phases are the initial phase, the plateau, and the slow recovery phase, shown to represent 16%, 53%, and 31%, respectively, of total EPOC in Atlantic salmon parr at 12 ºC (Zhang *et al.* 2018). These authors showed that the initial phase lasted 0.7h (42 min) and had the highest oxygen consumption (Zhang *et al*. 2018). These results can be generalized to a two-phase approach where approximately 20% of EPOC falls within the initial rapid recovery and the remaining 80% in the slow recovery phase.

Stress is a physiological response to an environmental variable know as a stressor (Schreck *et al*. 2016). Wild salmon are subjected to various stressors such as predator attacks, foraging, migration, and reproduction within their natural environment. An increase in MR is a fundamental outcome of the stress response and is needed to support recovery. Treberg *et al*. (2016) outlined the energy budget in a fish as energy in, out, and retained, indicating that a portion of the $energy_{(out)}$ is used to maintain homeostasis in the

fish and is termed basal costs. Chabot *et al.* (2016) SMR (see above) definition does not include the costs of routine activities. Treberg *et al*. (2016) RMR is an estimate of the MR that includes the routine activity of the fish. For this thesis, there were two stressors: the first is infection with ISAV. The second is chasing the fish for a fixed duration of time (see Materials and Method) for respirometry analysis. The response to the second stressor can be observed as an increase in MR above RMR.

To meet their metabolic demands, wild salmon must adapt to environmental stressors. By maintaining moderately elevated post-stress oxygen consumption with minimal impact on AMS, they can recover quickly (Farrell *et al*. 2003, Farrell 2006). EXPOC (Excess Post-Stress Oxygen Consumption) will be used as a proxy for EPOC since the latter refers to exercise stress rather than the handling and startling stress used (see Materials and Methods section). The maximum $MO_2$ in farmed Atlantic salmon is estimated to be 61.6 ± 6.6 mg/kg/h between 5 ºC and 15 ºC (Grøttum and Sigholt 1998). Should oxygen not be readily available, the aerobic swimming performance and metabolism will be reduced (Kutty and Saunders 1972).

### 1.9 Research objectives

This thesis focuses on the physiological costs of a sub-lethal ISAV HPRΔ infection in wild Atlantic salmon (F1 progeny of Tobique River origin) through its effects on aerobic metabolic rate, oxygen-carrying capacity of the blood, and protein synthesis rate. The thesis is empirical in nature with the main question being: is there a difference between wild fish infected with ISAV versus non-infected fish? Basic predictions used to address this question are: (1) the virus should generate some form of anemic response in the host

fish observable in one or perhaps all of the blood parameters quantified ((RBCC, hematocrit and hemoglobin concentration), (2) anemia should impact the metabolic rate as the host would have a reduced ability for oxygen transportation, and (3)) ISAV+ fish should have a higher rate of protein synthesis as their immune system would be responding to an infection.

Conducting this study required establishing the minimal infective dose (MID) for this virus strain when infecting wild-type salmon. MID is defined as the concentration of viruses needed to initiate an infection that generates clinical or sub-clinical sequelae (Ward *et al*. 1984, Yezli and Otter 2011). For this project, a sub-lethal infection was defined as 100% viral prevalence in fish detectable by real-time quantitative polymerase chain reaction (RT-qPCR). The host may (clinical infection) or may not (sub-clinical infection) show typical ISA symptoms. The relative viral load must be sub-lethal, causing no mortalities or mortalities that are no higher than those in the negative control fish. The focus is on the peak to post-peak infection window, when the sub-lethal viral load levels are highest.

Zhang *et al.* (2019) used intermittent stop-flow respirometry on individual fish to study the physiological cost of reovirus infection in salmon. Here, whole-tank intermittent stop-flow respirometry, a modification from Zhang *et al.* (2019), was used to quantify the metabolic costs of sub-lethal infection with ISAV on groups of wild salmon, as presented by Hvas and Oppedal (2019). Modification of protocols used for estimating AMS (Clark *et al*. 2013; Chabot 2016, Norin and Clark 2016, Halsey *et al*. 2018) were used to quantify routine metabolic rate (RMR), and modifications to EPOC (Lee *et al.*

2003a, 2003b) were used to accommodate the respirometer measurements of a group of fish here termed the EXPOC.

As the ISA virus replicates in the host's body, the number of erythrocytes should start to decline, thereby causing the characteristic anemia in the host associated with this disease. Anemia was assessed by measuring hematocrit, red blood cell count (RBCC), and total blood hemoglobin (Hb) concentration. Protein synthesis activity has been observed to increase as fish mount an innate immune response to a pathogen (Dettleff *et al.* 2017, Guo *et al.* 2019). Rates of protein synthesis from kidney tissue samples were measured using a modified flooding dose technique (Garlick *et al*. 1980; Lamarre *et al*. 2015). The *in vivo* flooding dose technique was used to measure the rate of protein synthesis in healthy farmed Atlantic salmon (Owen et al. 1999) and healthy Arctic charr (*Salvelinus alpinus*) (Lamarre *et al*. 2015). However, this study is the first to use kidney samples from wild-type Atlantic salmon that have been sub-lethally infected with ISAV, as kidney leukocytes have been observed to be important targets for ISAV (Dannevig *et al*. 1994).

# CHAPTER 2- MATERIALS AND METHODS

## 2.0 Animal Use Protocol

All fish handling followed Canadian Council on Animal Care (CCAC) guidelines and was approved by the DFO SABS Animal Care Committee under Animal Use Protocols (AUP) 18-04 for the respirometry analysis and AUP 19-31 for protein synthesis trials.

## 2-1 Fish source

Wild-type Atlantic salmon (*Salmo salar*) parr of Tobique River lineage and from the 2017 year-class were obtained from the DFO Mactaquac Biodiversity Facility, Kingsclear, NB. They were progeny of approximately 90 females and 90 males that were group spawned at the facility and they are therefore considered the $F_1$ generation of Tobique River wild salmon. . They were transferred in one trip to the DFO St. Andrews Biological Station (SABS) holding laboratory. Fish were held in $1m^3$ tanks (n=8 tanks; ~100 fish per tank) with water volume set at ~675 L via internal standpipe. Fish were sedated following DFO standard operating procedure with 0.05 g/L of MS-222 (tricaine methanesulfonate, Aqualife Syndel Canada®, Nanaimo BC, Canada) buffered with sodium bicarbonate (following DFO standard operating procedure) and weighed (average 69.15 ± 29.00 g standard deviation, all mean measurements hereafter follow this format) for an initial stocking density of 11 $kg/m^3$ ± 0.003 $kg/m^3$. Brackish water (5 ppt salinity) was pumped into a single head tank and passed through an ultraviolet (UV) sterilization chamber before being distributed to the individual tanks at a flow rate of one tank volume per hour. The water temperature regulation had set points between 7.5 and

8.5 ºC to maintain a mean of 8 ºC. The dissolved oxygen content of the water supplied to the tanks was monitored daily and kept within 80-100 % of air saturation. Each tank had an airlift and a ceramic bubble (oxygen) diffuser to ensure that oxygen levels did not go below 80 % air saturation. The laboratory was on an automatic 12 : 12 hour (day : night) photoperiod setting. Fish were fed once daily with size-appropriate commercial salmon grower pellets (Skretting, NB, Canada, 200EP 4mm) at 1 % of tank biomass. Clear plexiglass covers were placed over the tank lip. Based on DFO standard operating procedure, a one-hour formalin treatment (Parasite-S, Syndel, WA, USA), at a ratio of 1:4000, was applied for three consecutive days when fish were received as a routine preventive measure against fungal growth and other skin diseases. Thereafter, approximately every two weeks, the fish were exposed to a salt bath (full-strength sea water; 35ppt) for one hour to prevent fungus and other pathogens from growing. The water source at the head tank was changed from brackish to full-strength sea waterwater for this treatment and then back to brackish.

Once the fish had grown to ~100 g and parr markings had faded, they were ready for the smoltification process. The photoperiod was set to continuous light, salinity was increased by five ppt every two days, up to full-strength sea water over two weeks, and the temperature was raised from 10 ºC ± 0.5 ºC to 12 ºC ± 0.5 ºC at a rate of 1 ºC over two days. Salinity was kept at full-strength sea water for the remainder of the study. After completion of the smoltification process, fish were moved from the holding laboratory to the biocontainment facility. Once moved, they were maintained at 12 ºC ± 0.5 ºC, a 12:12 photoperiod and in full-strength sea water. Fish tanks were cleaned and monitored daily

for signs of disease, dissolved oxygen levels, and water temperature. Dissolved oxygen levels were maintained above 80% air saturation. Fish were fed once daily.

### 2-2-0 Viral Strains

Strain ISAV CA/NS/2012-21/2012 (hereafter referred to as NS/2012) isolated from an outbreak in Nova Scotia (LeBlanc *et al*. 2018) was chosen for this challenge. Fish from the original outbreak showed characteristic symptoms of ISA including mortality. The strain genotype is H17.11 NA based on Gagné and Leblanc (2017). The virus was multiplied from frozen aliquots on ASK cells before the challenges. The viral culture was prepared at the DFO Gulf Fisheries Centre's Molecular Biology Unit (DFO-GFC-MBU) using dilutions in Hank's buffered salt solution (HBSS) of a stock culture with an initial titer of $10^{6.5}$ $TCID_{50}$/mL (Table 1). Viral titers were calculated using the Spearman-Karber method (Reed and Muench 1938).

### 2-2-1 Relative Viral Load.

All challenges (MID, respirometry, and protein synthesis experiments) used RT-qPCR and mean Critical Threshold values (Ct-Values) to initially quantify the virus from blood samples (see section 2-4-0). Relative viral load was calculated following methods outlined by Leblanc *et al.* 2018. RT-qPCR assays were done using specific primers, probes, and conditions optimised for ISAV detection as described in LeBlanc *et al.* 2018. The samples were considered negative if no amplification was observed after 40 cycles of thermocycling. Thus no Ct (no amplification) was converted to a value of 40 when applicable.

## 2-3 ISAV Challenges

### 2-3-1 Minimal Infectious Dose (MID)

The minimal infectious dose (MID) challenge was completed when the fish were 174.9 ± 16.2 g. The initial plan for infecting fish for the respirometry and protein synthesis analyses was to use bath immersion, which required determination of the MID to ensure a sub-lethal exposure to the virus. Twelve fiberglass 1 $m^3$ tanks (as mentioned above) in the SABS biocontainment laboratory were each fitted with a 0.275 $m^3$ fiberglass insert, a spray bar for water input, an air stone, a ceramic oxygen diffuser, an optical oxygen sensor (RDO PRO-X optical probe, In-SityInc®) and a transparent Plexiglas cover. Twenty fish were distributed randomly to each of the 12 tanks and fasted for 24 hours before the challenge.

Before exposure to the virus, 20 naïve fish were selected: two from each of the tanks from tank 2 through tank 11. They were anesthetized (MS-222 at 0.05 mg/L), and blood (4-6 drops of approx. 0.05 mL) was collected non-lethally from the caudal vasculature using a non-heparinized hypodermic syringe and stored in 1 mL Trizol (Life Technologies Tri reagent®) to screen for pre-exposure to ISAV by RT-qPCR at the DFO-GFC-MBU (see section 2.4 Lab Analysis for procedure). Once blood was taken, the fish were returned to their respective tanks.

Fish were exposed to the virus via bath challenge for one hour by transferring 20 fish to each of 12 clear plastic bins containing 40 L of UV-sterilized seawater with the pre-determined concentration of ISAV (Table 1). Each bath had an air stone and ceramic oxygen diffuser. Aeration was turned on before introducing fish, and ceramic diffusers were used to infuse oxygen should the concentration drop below 80 % air saturation

measured by a handheld optical dissolved oxygen meter (YSI 6050020 Pro 20). Baths were covered with clear plastic lids for the bath exposure duration. Negative and positive controls contained only sterile Hank's Buffered Salt Solution (HBSS) and a high viral concentration, respectively. Fish were returned to their respective home tanks following treatment. At 21, 35, and 67 days post-infection (DPI), fish (n = 5/tank) were transferred to a sedation bath of MS-222 (0.05 mg/L) and their body mass measured. Blood samples (0.5 mL) were collected as described above and processed by RT-qPCR at the DFO-GFC-MBU following the procedure outlined in section 2-4-1 to assess Ct-values. Mean Ct was used to calculate mean relative viral loads (Section 2-2-1).

**Table 1:** Experimental set-up for the 1-hour bath exposure challenge with NS/2012-21 in 40L plastic bins (20 fish/bin). All viral concentrations were prepared from a stock culture of $10^{6.5}$ $TCID_{50}$/mL.

| Bin # | Viral concentration ($TCID_{50}$/mL) |
|---|---|
| 1 | 0 (Negative control) |
| 2 | 0.016 |
| 3 | 0.016 |
| 4 | 0.08 |
| 5 | 0.08 |
| 6 | 0.4 |
| 7 | 0.4 |
| 8 | 2 |
| 9 | 2 |
| 10 | 10 |
| 11 | 10 |
| 12 | $10^3$ (positive control) |

### 2-3-2 Respirometry

The respirometry and protein synthesis analyses were initially planned to use fish infected by immersion to reflect a natural route of infection. However, the ISAV strain did not cause mortality during the MID bath challenge described above (2-3-1), and therefore a cohabitation method was attempted. Fish were transferred to the DFO SABS biocontainment laboratory (mean body mass 261.3 g ± 5.2 g) and screened for pre-challenge exposure to ISAV using RT-qPCR (see section 2.4 lab analysis). These fish were kept at a mean density of 28.7 kg/$m^3$ ± 0.1 kg/$m^3$ within the 0.275 $m^3$ inserts (n=6). Trojan and sham fish were injected with either 0.1 mL of MID dose or 0.1 mL of sterile HBSS, respectively, adipose fin-clipped, and placed in separate holding tanks away from naïve fish. At 21 DPI, five Trojans were moved to each of three tanks of naïve fish, and five sham-injected fish were moved to each of three different tanks of naïve fish. Trojans and shams were removed and euthanized after seven days of cohabitation with naïve fish. Blood samples were taken and screened for viral load by RT-qPCR to confirm infection. Trojan fish were all positive for infection; however, cohabitant fish remained negative.

All remaining fish (see table 2) in the Trojan-exposed tanks were intraperitoneally (IP) -injected with 0.1 mL of NS/2012 at $10^5$ TCID50/mL following the procedure outlined by LeBlanc *et al*. (2018), and those in the control-exposed tanks were IP-injected with 0.1 mL of sterile HBSS. At two weeks post-injection, five fish/tank were blood sampled, and RT-qPCR screened to confirm infections.

*Tank set up:*

All respirometry analysis was completed in the same system described above. The head tank was regulated to have oxygen at 120 % air saturation. Each experimental tank was equipped with an optical probe (routinely calibrated to ensure accurate readings) that would signal the tank's solenoid to open when air saturation levels were at 80 %, ensuring that all tanks had sufficient oxygen levels for the fish. Six holding tanks housed the fish when they were not in the tanks designated for respirometry measurements.

Fish spent most of their time in holding tanks and were transferred to their corresponding respirometry tanks 24-48 hours before challenge day. Six tanks were prepared for respirometry experiments by adding a submersible pump, following a Svendsen *et al*. (2016) tank design. Tanks had plexiglass covers placed over them before respirometry measurements as described below. The head tank was equipped with an additional optical oxygen probe, an oxygen diffuser at the intake pipe, and four circulation pumps (the same as listed above) as mixing water is essential for uniform distribution (Rodgers *et al*. 2016). The optical oxygen probes were placed at a 90° angle relative to the ceramic oxygen diffuser in all tanks and were set to collect readings once every minute. Water flowed from the head tank to the UV sterilizer before being distributed to individual tanks. The water temperature was held at 12.0 $^{o}$C ± 0.5 $^{o}$C.

*Stress challenge and intermittent stop-flow respirometry:*

Respirometry of stress-challenged fish was performed twice, at 16 and 30 DPI. Stressor here will be referring to the 2 min chasing hereafter. Fish were netted from their home tanks and transferred to their respective respirometry tanks 48 hours pre-challenge.

Fish fasted for 24 hours pre-challenge. Table 2 shows the body mass and stocking density of fish in each tank for the two challenges. Technicians did not enter the laboratory once fish had been transferred to their respective respirometry tanks. Technicians returned for the start of the stress challenge. The head tank dissolved oxygen was recorded as percent saturation and mg/L at intervals of one min for the duration of all respirometry analysis. In addition to routine monthly calibration, optical oxygen probes were calibrated before the start of each respirometry experiments using the manufacturer's instructions.

Pre-stress oxygen consumption rates (RMR) were measured as soon as instrument calibration was completed. Air stones were removed from tanks, while oxygen diffusers were left in tanks. These diffusers were turned on only when prompted by the programmed set point of 80 % dissolved oxygen saturation. Lastly, water flow was shut off at the tank intake valves.

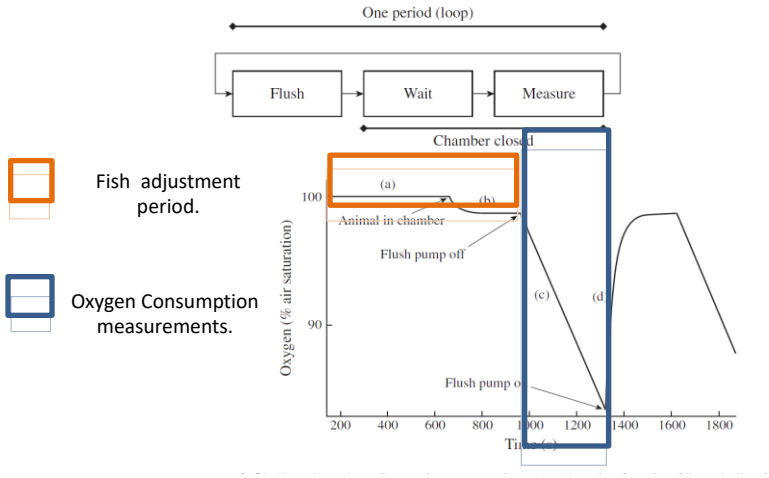
Respirometry followed a modified version of Svendsen *et al*. (2016) intermittent stop-flow respirometry loop (Figure 1), using the whole tank as a respirometer for the entire group of fish rather than individuals within a smaller respirometer. There was also no water flushing to reoxygenate the water while fish were recovering from their stress; instead, water was reoxygenated using the oxygen diffuser (Figure 2).

Ammonia build-up was of minor concern in this study as fish were not fed 24 hours prior to the start of respirometry, and ammonia production peaks 4-6 hours post-feeding in farmed post-smolt Atlantic salmon and excretion rates vary depending on feeding frequency (Forsberg 1995). Wakefield *et al.* (2004) observed salmon fry to produce 0.45 μmol/g/h and three times this amount post-exercise, so there will undoubtedly be a minor build-up post respirometry challenges. The $CO_2$ levels were not of primary concern here

as fish densities were held at a maximum of 28.2 ± 1 kg/$m^3$ for an average time of 50 min in a closed tank at 12 ± 0.5 ºC. Adult Atlantic salmon at a density of 70 kg/$m^3$ have been observed to take 56 min to produce the sub-lethal levels of $CO_2$ (19.6 mg/L) in 10.6 ± 1.2 ºC seawater (Tang *et al.* 2009). Stocking densities for respirometry were approximately a third of those observed by Tang *et al.* (2009). Oxygen levels (as % air saturation) were logged at one-minute intervals with the optical probes; this measurement was deemed acceptable as Zhang *et al.* (2018) describe the first recovery to be 42 min for Atlantic salmon at 12 ºC.

Once the first cycle of pre-stress oxygen consumption was completed, Plexiglas covers were removed, and the fish were chased by two individuals using two nets for two minutes to stress them (Cooke *et al.* 2015). The Plexiglas cover was then put back in place, and oxygen uptake was measured. As above, automated solenoid valves were programmed to turn the oxygen on when dissolved oxygen fell to 80% and off when it had returned to ~ 110 %. A manual relief valve was used to bleed excess oxygen from the line when the valves closed. These cycles were timed, and respirometry was completed when a tank took the same amount of time to reach 80 % oxygen saturation mark as its pre-stress time for two consecutive cycles.

Biofilm oxygen consumption of the respirometry tanks (i.e., without fish) was measured overnight and into the morning using the SABS Delta system's data and then subtracted from the original fish oxygen consumption data for each tank in Excel. The $\Delta C_{wo2}$ values of formula (1) were calculated in mg/L in accordance with Clark *et al*. (2013) and Wood (2018). Temperature, water salinity, and barometric pressure were entered during optical probe calibration before respirometry analysis.




from Svendensen *et al* 2016

**Figure 1:** Theoretical and graphic representation of an intermittent respirometry loop as conceived by Svendsen *et al*. (2016). Phases a and b were combined for the respirometry analysis (red box). Modification of this protocol happened at the start and end of phase c (blue box): water was reoxygenated by delivery of compressed oxygen using a ceramic bubble diffuser.

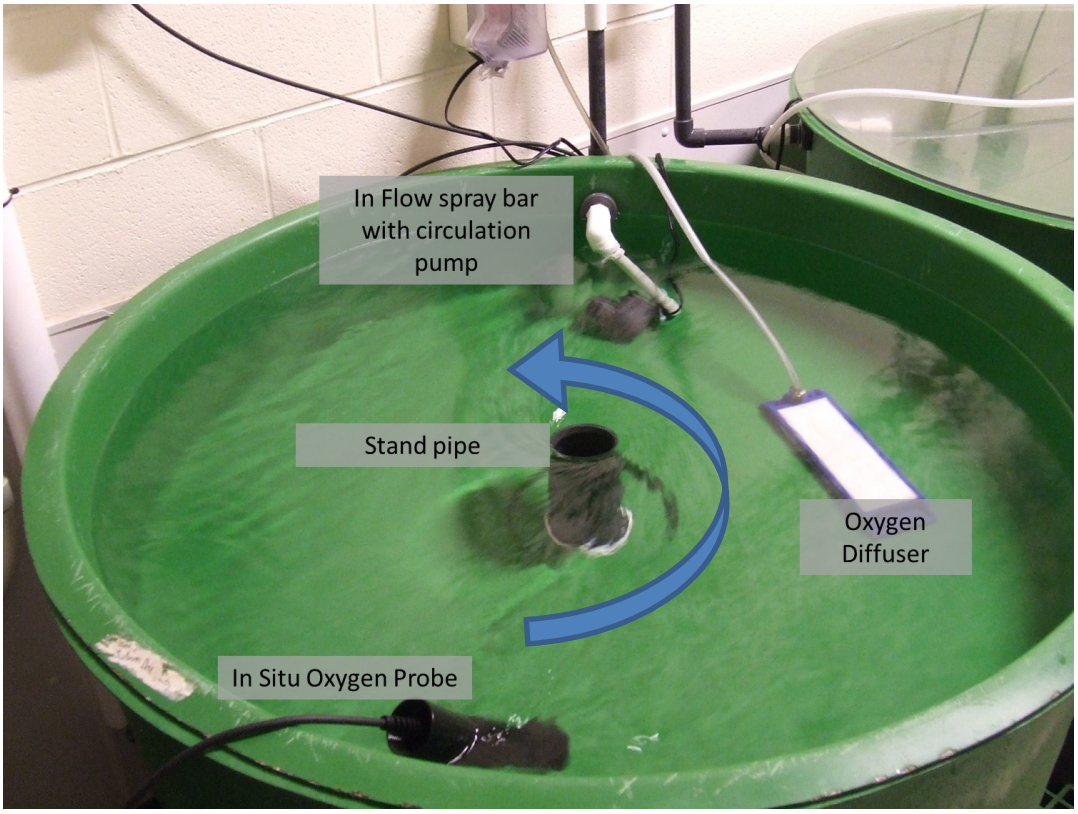



**Figure 2:** Experimental set-up of respirometry tank. The insert shown (0.275 $m^3$) was placed inside a 1-$m^3$ tank to reduce the water volume. An optical oxygen probe was set up at 90° from the ceramic oxygen diffuser and 180° from the water in-flow and circulation pump. The blue arrow shows the direction of water flow. A clear Plexiglass cover was placed over the tank lip to prevent fish escape (not shown), and water was added to fill as much of the space under the cover as possible.

*Metabolism terminology and calculations:*

$MO_2$ was calculated using the formula (Clark *et al*. 2013 and Wood 2018):

(1) $MO_2$ (mg/kg/hour) = $[(V_r-V_f) \times \Delta C_{wo2}] / (\Delta t \times M_f)$

$V_r$ = Respirometer tank volume (L)

$V_f$ = Volume of water displaced by fish in respirometer tank (L; measured after the analysis)

$\Delta C_{wo2}$ = Change in the oxygen concentration during the stop-flow phase (mg/L)

$\Delta t$ = Duration of $\Delta C_{wo2}$ measurement (hour)

$M_f$ = fish body mass (kg)

Since the experimental approach did not allow for the measurement of true SMR (because fish were not inactive and in a fully post-absorptive state) or MMR, it was impossible to calculate true AMS. Instead, the pre-stress $MO_2$ was used as a proxy for routine metabolic rate (RMR) and the first post-stress cycle $MO_2$ (referred to as PC1) as a proxy for MMR to calculate a post-stress absolute aerobic metabolic scope (PSAMS):

(2) PSAMS (mg/kg/hour) = Mean of all PC1 $MO_2$ – Mean of all Pre-stress $MO_2$

Plotting oxygen concentration against time made it possible to observe a respirometry cycle curve matching the description of Svendsen *et al*. 2016 (Figure 1) with the blue squared area under the oxygen consumption curve representing the general area used for calculations of EXPOC (See Appendix for details). This value was determined by

integrating the area under the $MO_2$ curve (Zhang *et al*. 2018, 2019). Here the readings of oxygen from the water were converted to $MO_2$ values. These were grouped into 5 min intervals and plotted. The RMR (mean of pre-stress oxygen consumption) was plotted and 5 % above this value (see Appendix Table 1) used as a reference point to delineate the zone of 20 % rapid phase recovery of the fish as outlined by Zhang *et al.* (2018).. This rapid phase was found in the steepest part of the curve within the first hour of recovery (Brett and Glass 1973, Thorarensen *et al*. 1996, Gallaugher *et al*. 2001). Once the sections of the rapid phase recovery $MO_2$ were established, it was then possible to calculate the area under each respective curve using its integral (Zhang *et al.* 2018, Zhang *et al.* 2019). This results in the approximation of EPOC but termed EXPOC for reasons explained above.

*Post-respirometry sampling:*

Once pre- and post-stress $MO_2$ measurements were completed, blood samples were collected from five fish per tank (as per section 2-3-1) for ISAV RT-qPCR (section 2-4-1). Mean Ct was used as a proxy for mean relative viral loads (Section 2-2-1). The remaining blood was placed in 1.5 mL microcentrifuge tubes containing 10 µL of heparin (140 units/mg, 0.1mg/mL heparin sodium salt Sigma-Aldrich H-0777, Sigma-Aldrich Corp St. Louis, MO USA in $ddH_2O$ as per MacLatchy *et al.*, 2005) for red blood cell count (RBCC), hematocrit, and hemoglobin concentration determination as described in section 2-4.

**Table 2:** Fish body mass (mean ± standard deviation) and stocking density at 16 and 30 days post-injection (DPI) in the respirometry analysis. Tanks 1, 2, and 5 were the control (HBSS-injected), and tanks 3, 4, and 6, the ISAV-positive tanks. Fish were individually measured after each analysis. The total biomass (kg) was used to calculate the stocking density of fish within the tanks, with volume corrected for water displaced by the fish. The number of fish decreased in tanks 2, 5 and 6 between dates because fish were removed to ensure tank density was similar. There was no mortality between dates.

| 16 DPI | | | | 30 DPI | | | |
|---|---|---|---|---|---|---|---|
| Tank Number | Number of Fish | Body Mass (kg) | Density (kg/m³) | Tank Number | Number of Fish | Body Mass (kg) | Density (kg/m³) |
| 1 | 30 | 0.25 ± 0.06 | 27.1 | 1 | 30 | 0.26 ± 0.07 | 28.3 |
| 2 | 33 | 0.26 ± 0.07 | 28.1 | 2 | 32 | 0.27 ± 0.07 | 31.2 |
| 3 | 30 | 0.26 ± 0.07 | 27.4 | 3 | 30 | 0.27 ± 0.07 | 29.7 |
| 4 | 31 | 0.24 ± 0.07 | 25.5 | 4 | 31 | 0.26 ± 0.08 | 29.1 |
| 5 | 29 | 0.25 ± 0.07 | 26.1 | 5 | 25 | 0.27 ± 0.08 | 24.3 |
| 6 | 29 | 0.25 ± 0.09 | 26.2 | 6 | 26 | 0.27 ± 0.08 | 25.7 |

### 2-3-3 Protein Synthesis

*Fish and experimental set-up:*

For the protein synthesis analysis, another group of fish were screened pre-challenge using RT-qPCR for previous exposure to ISAV. Thirty fish were placed in each of the six tanks. At the start of the challenge, the fish had a mean body mass of 350.0 ± 48.0 g. Fish in three tanks were IP-injected with 0.1 mL of ISAV NS 2012 at $10^5$ $TCID_{50}$/mL, and those in the other three tanks were IP-injected with 0.1 mL of sterile HBSS (control groups). The head tank and experimental tanks were set up as described in the respirometry section.

*Sampling:*

The protein synthesis measurements were done at 7, 17, and 78 DPI, with feed withheld 24 hours before sampling. Individual fish were randomly selected (n=5 per tank), weighed, injected with 0.1 mL/kg of 50 % ring-D5-L-phenylalanine (Cambridge Isotope Laboratories), and placed in a separate tank for an incubation period of 116 min ± 4 min, following Lamarre *et al.* (2015) protocols, before lethal sampling. Kidney samples were collected (50-100 mg per fish), flash-frozen in liquid nitrogen, and stored at – 80 °C until processing at l'Université de Moncton, Moncton, NB, using the flooding dose technique for determining the rate of protein synthesis (Lamarre *et al.* 2015). Blood samples were collected (section 2-3-1) before necropsy for ISAV RT-qPCR (section 2-4-1), and mean Ct values were used to calculate mean relative viral loads (Section 2-2-1). The remaining blood was placed into a heparin-coated 1.5 mL centrifuge tube for RBCC, hematocrit, and hemoglobin concentration determination as described below (section 2-4).

*Sample Dates Respirometry vs Protein Synthesis:*

Sample dates in the respirometry analysis were 16 DPI and 30 DPI, whereas, in the protein synthesis analysis, they were 7, 17, and 78 DPI. ISAV takes two weeks to reach peak infection. The respirometry analysis was aimed to capture peak infection (approximately 14 DPI) and then its decline (30DPI), as these were hypothesized to be the most significant times during the infection cycle. Protein synthesis is a critical process to growth and in response to infection. Protein synthesis rate sample dates were different from the sample dates of respirometry to capture a more complete picture of ISAV infection progression, from the initial phase, through the peak phase to the recovery phase of the fish, and these dates were chosen as 7, 17, and 78 DPI, respectively.

## 2-4 Analytical Techniques

### 2-4-0 ISAV detection by RT-qPCR

The DFO-GFC-MBU completed RT-qPCR analysis for ISAV. Total RNA was extracted from approximately 0.1 mL blood using TRI reagent® solution following manufacturer's instructions and quantified at 260 nm using a plate reader spectrophotometer (Multiskan™ GO; Thermo Fisher Scientific, Massachusetts, USA). Extracts were then normalized at 150 ng/µL, and 1500 ng were reverse transcribed using the High Capacity cDNA RT kit (Applied Biosystems™, Foster City, CA, USA) following the manufacturer's instructions. Detection of ISAV segment 8 was then done by real-time PCR using 12.5 µL TaqMan Universal master mix (Applied Biosystems™, Foster City, CA, USA), 2 µL prepared cDNA as template (~ 75 ng cDNA), 0.5 µM 404F primer (5'-TGGGCAATGGTGTATGGTATGA-3'), 0.5 µM 583R primer (5'

GAAGTCGATGAACTGCAGCGA-3') and 0.2 µM FAM-labeled probe (5'-CAGGATGCAGATGTATGC-3') in a total volume of 25 µL. PCR conditions were 2 min at 50 °C, 10 min at 95 °C, and then 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Probes were obtained from Thermo Fisher Scientific (Massachusetts, USA) and primers from Sigma-Aldrich (Ontario, Canada). Ct values obtained from serially diluted *in vitro* transcribed RNA with ISAV sequences were used to obtain standard curves and calculate RNA viral copy numbers.

### 2-4-1 Hematocrit determination

Blood was drawn from sample tubes into capillary glass tubes, sealed with putty at one end, and centrifuged (IEC CL3® series centrifuge, Thermo Electron Corporation, with Hemato-Kit Rotor 930) at 3500 rpm for 10 min as per manufacturer instructions. Hematocrit (one sample per fish) was determined by measuring the height of the red blood cells (RBC) and total blood volume in the capillary tubes and then using the formula:

$$\textbf{(3)}\ Hematocrit\ (\%) = \frac{\text{RBCC height (mm)}}{\text{total blood height (mm)}}\ \text{x}\ 100$$

### 2-4-2 Hemoglobin concentration

Blood was placed in heparinized microcentrifuge tubes as described above (section 2-3-2) and frozen at -20 ºC until analysis. Hemoglobin measurement was completed in the DFO-GFC-MBU Lab using the cyanmethemoglobin method (Sandnes *et al.* 1988). Standard human hemoglobin (SHHb) (10 mg/mL) was prepared from Sigma-Aldrich Human Hemoglobin Lot # SLBX 7566 and stored at 4 ºC. Drabkin's solution was

prepared using Drabkin's Reagent and 500 µL of Brij® L23 Solution (Sigma-Aldrich) following the manufacturer's instructions. A 1:2 serial dilution of SHHb was used to generate standard curves (one for each 96-well plate). Fish blood samples were analyzed at 1/20 dilution (duplicates) and were read on a spectrophotometer (Multiskan GO, ThermoFisher) at 540 nm.

### 2-4-3 Red blood cell count (RBCC)

Blood was placed in heparinized microtubes described above (section 2-3-2) and stored with heparin (as described above) until analysis. RBCC was determined using a 0.9 % saline solution for serial dilution (1000-fold). Twelve µL were placed onto each of two counting grids of a Neubauer hemocytometer and viewed with a compound microscope of the last dilution. Cells were counted in the five sub-squares of the center grid at 400x magnification. The mean of the two grid counts was used to calculate RBCC using the formula:

$$(4)\ \text{RBCC (cells/mm}^3) = \frac{\text{Mean RBCC count x 1000}}{\text{Total volume (mm}^3)}$$

Mean RBCC count = sum of the five square top grid count and the five square bottom grid count divided by two.

Total volume = total volume of the hemocytometer grid (0.02 $mm^3$).

### 2-4-4 Fractional rate of protein synthesis

*Homogenization*

After tissue collection, individually weighed kidney samples (average 70.29 ± 11.41 mg) were homogenized using 0.2 M perchloric acid (PCA) followed by 10 min incubation on ice and centrifugation at 15000 g for 5 min at 4 ºC. The supernatant (i.e., free-pool amino acids) was removed and stored at -20 ºC. The protein pellet was re-suspended in 1 mL 0.2M PCA, sonicated until tissue solutions were homogenous, and centrifuged at 15 000 g for 5 min at 4 ºC. This was repeated three times. The pellet was re-suspended in 1mL acetone using sonication and then centrifuged at 15 000 g for 5 min at 4 ºC. The supernatant was discarded. Glass tubes containing 6 mL of 6 M HCL received the washed (1 mL acetone and centrifuged 15 000 g for 5 min) protein pellet ready for hydrolysis. Glass tubes were incubated at 110 ºC for 16-18 hours.

*Solid Phase Extraction*

Phenylalanine extraction was performed in both supernatant and hydrolyzed pellet. Before extraction in the supernatant, free-pool samples received 100 µL of 5 M KOH, allowing 3 min for perchlorate to precipitate. Then, 100 µL of 6 M HCl was added and incubated for 3 min before a quick spin. The supernatant was used in solid-phase extraction (SPE) columns, i.e., phenylalanine extraction was completed using a C18 SPE cartridge (Sigma-Aldrich) rinsed with 1 mL methanol and 1mL 1 M HCl. Amino acids were eluted with 250 µL of 30% methanol. Eluates were dried in heating tubes for one hour at 110 ºC. Once dried, these pellets were solubilized in 75µL of distilled water; 50 µL of this solution was used for derivatization.

*Derivatization*

Following the extraction and evaporation of both types of samples, the amino acids were derivatized using pentafluorobenzyl bromide (PFBBr) in acetone and a 60 ºC

incubation for 45 min. Derivatized amino acids were extracted with n-hexane. The supernatant was removed and preserved.

The gas chromatography-mass spectrometry (GC-MS) (Agilent gas chromatograph, Model 6890N) was injected with 1 μL of n-hexane, and samples were then passed through for analysis. The chromatography column was a Zebron ZB-5MS capillary GC (Phenomenex Inc., 30 m x 0.25 mm x 0.30 μm). The initial temperature was 70 ºC and was increased at a rate of 25 ºC per min to 300 ºC. The total run time was 20 min. Fractional rates of protein synthesis (Ks, %/day) calculations were following the equations from Lamarre *et al*. (2015):

(5) Enrichment Protein Pool Supernatant:

$$S_b = \frac{D_5 - PHE}{PHE + (D_5 - PHE)}$$

D5-PHE = marked amino acid Ring 5D phenylalanine injected into the tissue

PHE = phenylalanine found in the fish tissue

(6) Hydrolyzed Protein Pellet and Free Amino acid Pool:

$$S_a = \frac{D_5 - PHE}{PHE + (D_5 - PHE)}$$

(7) Protein Synthesis Rate:

$$\text{Ks (\%/day)} = \frac{S_{b(t2)} - S_{b(t1)}}{S_a(t2 - t1)} \times \frac{1440}{t2 - t1} \text{ x } 100$$

$S_{b(t2)}$ = Final Protein-bound

$$D_5\text{-PHE: } \frac{D5 - PHE}{PHE + (D5 - PHE)}$$

$S_{b(t1)}$ = Average incorporation at initial time

$S_{a(t2 - t1)}$ = Average enrichment in the free pool between t2 and t1

t = Time measured in days

**2-5 Statistical analysis**

R-Studio was used to complete all statistical analyses. For the respirometry analysis, the response variables PSAMS, EXPOC, and $MO_2$ were analyzed using a 2-way repeated-measures analysis of variance (ANOVA) with an *a-priori* cut-off at $p < 0.05$ for statistical significance and using Tukey's Honestly Significant Difference tests for *post-hoc* analyses. Data collected for relative viral loads in the respirometer analysis did not meet the parameters of the two-way ANOVA test here; a t-test was performed to compare both ISAV-injected fish relative viral loads. RBCC, hematocrit, and hemoglobin values were analyzed using 2-way ANOVA with an *a-priori* cut-off at $p < 0.05$ for statistical significance and using Tukey's Honestly Significant Difference tests for *post-hoc* analyses. Pearson's correlation coefficient test was used to assess the strength of the correlation between relative viral loads and the response variables RBCC, hematocrit, and hemoglobin.

The protein synthesis rate analysis variables (RBCC, hematocrit, hemoglobin, and Ks) were also compared between treatments (ISAV and sham injection) and sampling dates (7, 17, and 78 DPI) using separate 2-way ANOVAs (*a-priori* $p < 0.05$ and *post-hoc* Tukey Honestly Significant Difference tests). Data collected did not meet the parameters of the two-way ANOVA test here; a one-way ANOVA was performed to compare both ISAV-injected fish relative viral loads (*a-priori* $p < 0.05$ and *post-hoc* Tukey Honestly Significant Difference tests). Pearson's correlation coefficient test was used to assess the

strength of the correlation between relative viral loads and the response variables RBCC, hematocrit, hemoglobin, and Ks.

# CHAPTER 3- RESULTS

## 3-1 Fish Body Mass and Mortality

There was no significant difference in body mass of fish between the respirometry and protein synthesis experiments ($p = 0.06$). There was a significant difference observed in the body mass of the MID fish vs that of the protein synthesis fish ($p = 0.01$) (See Appendix: Table 2 for fish numbers and stocking densities). Fish have been injected with an equal dose of virus, without taking into consideration the variation of body mass. There were no mortalities associated with ISAV infection during any of the respirometry or protein synthesis experiments.

## 3-2 Minimal Infectious Dose

At 21 DPI, the positive control tank (1000 $TCID_{50}$/mL) had 100 % prevalence of ISAV (Figure 3). Samples from 35 DPI were improperly stored and could not be analyzed. At 67 DPI, the positive control tank remained at 100 % prevalence of infection, and one of the replicate tanks at 10 $TCID_{50}$/mL was also at 100 % prevalence. The viral dose that satisfied all the sub-lethal criteria (i.e., 100 % viral prevalence and no symptoms of ISAV) chosen as the MID was 0.1 mL of $10^3$ $TCID_{50}$/mL. However, this dose was higher than expected. Fish did not show symptoms of ISAV, although they were infected.

## 3-3 Respirometry Results

*Infection:*

Although the Trojans had a confirmed viremia with RT-qPCR at 14 DPI (mean Ct = 22.82 ± 2.56) before being added with naïve fish, they failed to infect naïve fish after seven days of cohabitation. IP injection of individual fish with a dose of 0.1 mL $10^5$ $TCID_{50}$/mL of ISAV (Leblanc *et al.* 2018) was used to ensure that all fish were infected with ISAV. Viremia was confirmed with RT-qPCR at 14 DPI (mean Ct = 23.13 ± 3.20).

Infection was observed in the fish used for respirometry analysis for ISAV+ IP injected fish. (Figure 4). T-test results indicated no significant difference was observed between 16 DPI and 30 DPI (t-value = 0.71, p = 0.48) in the ISAV infection levels. Sham-injected fish samples had no detectable infection after 40 cycles in RT-qPCR.

*Oxygen consumption parameters:*

Figures 5 and 6 show raw traces of dissolved oxygen decline due to fish respiration in tank water used to calculate the $MO_2$ values. These figures also indicate the time taken to complete each cycle (PRE, PC 1 through PC 4) shown in figure 8. None of the measures of aerobic metabolism (PSAMS, EXPOC, mean pre-stress and all post-stress $MO_2$ values) were affected by viral load (sham- versus ISAV-injected), time (16 or 30 DPI), or their interaction (Figures 7, 8, and 9, Table 3). Mean values for PSAMS were within the range of 90.70 ± 25 $mgO_2$/kg/h to 107.71 ± 31 $mgO_2$/kg/h for control and ISAV+ during both sample dates, no significant differences were observed. Mean EXPOC values were in the range of 30-5050 $mgO_2$/kg, with no effect of treatment (control versus ISAV+) or sampling date (Figure 9, Table 3)).

It is important to note that at 16 DPI there was a malfunction with tank 1. The optical probe was too close to the oxygen diffuser causing false water air saturation readings. This tank was excluded from analysis.

*Blood Parameters:*

RBCC values were not affected by viral load but increased over time, although control fish were no different from ISAV infected fish. (Figure 10, Table 3). *Post-hoc* analysis showed that 30 DPI sham-injected ($p = 0.02$) and ISAV+ ($p < 0.01$) fish had significantly higher values (2226667 ± 402167 cells/mm$^3$, 2713333 ± 184891 cells/mm$^3$, respectively) than corresponding sham-injected and ISAV+ fish at 16 DPI (1920000 ± 196442 cells/mm$^3$, 1695000 ± 295985 cells/mm$^3$ (16 DPI ISAV+, respectively). Hematocrit values were higher at 30 DPI than at 16 DPI but were not affected by viral load or the interaction between DPI and viral load (Figure 11, Table 3). Hemoglobin was observed to have no significant interaction between time and viral load (Figure 12, Table 3) with a range of 16.76 ± 1.76/dL to 19.06 ± 2.75g/dL. No significant correlations were found between relative viral loads and any of these blood parameters (Table 5).

**3-4 Protein Synthesis**

*Infection:*

Treatment (sham- versus ISAV-injected), time after treatment, and their interaction all had a significant effect on viral load (Figure 13, Table 4). One way ANOVA *Post-hoc* analysis indicated that CT-values increased significantly from 7 DPI to 17 DPI ($10^4 \pm 10^{0.59}$ and $10^5 \pm 10^{0.81}$, respectively; $p < 0.0001$) and then decreased significantly from 17 DPI to 78 DPI ($10^{3.08} \pm 10^{0.45}$; $p < 0.001$) and were significantly lower at 78 DPI than 7

DPI ($p < 0.0001$). Sham-injected fish samples had no detectable infection after 40 cycles in RT-qPCR.

*Protein Synthesis Rates:*

Protein synthesis rate was affected by treatment, time, and their interaction (Figure 14, Table 4). *Post-hoc* analysis indicated that Ks values were significantly higher in ISAV+ fish than in sham-injected fish at 7 DPI (by 35.8 %, i.e., 7.51 ± 1.75 %/day versus 5.53 ± 1.62 %/day, respectively; $p = 0.001$) and at 17 DPI (by 15.7 %, i.e., 7.65 ± 1.73 %/day versus 6.61 ± 1.10 %/day, respectively; $p = 0.06$), but not at 78 DPI (5.46 ± 0.89 %/day (ISAV+) versus 5.14 ± 0.74 %/day, respectively; $p = 0.973$). The Ks values for ISAV+ fish at 78 DPI were significantly lower than at 7 DPI ($p = 0.002$) and 17 DPI ($p = 0.003$). There was a significant positive correlation (0.45) between relative viral loads and protein synthesis rates ($p < 0.0001$; Table 5). Sham-injected fish samples had no detectable infection after 40 cycles in RT-qPCR.

*Blood Parameters:*

RBCC was not affected by time but was significantly lower in ISAV+ fish than in sham-injected controls at 17 DPI (2041667 ± 187003 cells/$mm^3$, 2560000 ± 267562 cells/$mm^3$, respectively $p < 0.0001$), and there was a significant interaction between time and treatment (Figure 15 Table 4). *Post-hoc* analysis showed that RBCC values of ISAV+ fish were only significantly different from sham-injected fish at 17 DPI (10% lower; $p = 1.6 \times 10^{-5}$ ).Hematocrit was affected by time, treatment, and their interaction (Figure 16 and Table 4). *Post-hoc* analysis showed hematocrit values of sham-injected fish were only significantly different from ISAV+ fish at 7 DPI (56.22 ± 5 % and 47.88 ± 4 %, respectively; $p = 9.8 \times 10^{-5}$). Hemoglobin values were not affected by ISAV

treatment but were affected by time, with a significant interaction between time and treatment (Figure 17, Table 4). The *post-hoc* analysis only found a significant difference ($p=0.007$) in hemoglobin values between sham-injected fish at 7 DPI (17.04 ± 1.81 g/dL) and 17 DPI (14.60 ± 1.16 g/dL). Hemoglobin values at 7 DPI were 7 % lower in ISAV+ fish (15.51 ± 1.41g/dL) compared to the sham-injected controls at this time ($p = 0.0007$) There was a significant negative correlation between relative viral loads and all blood parameters (Table 5).

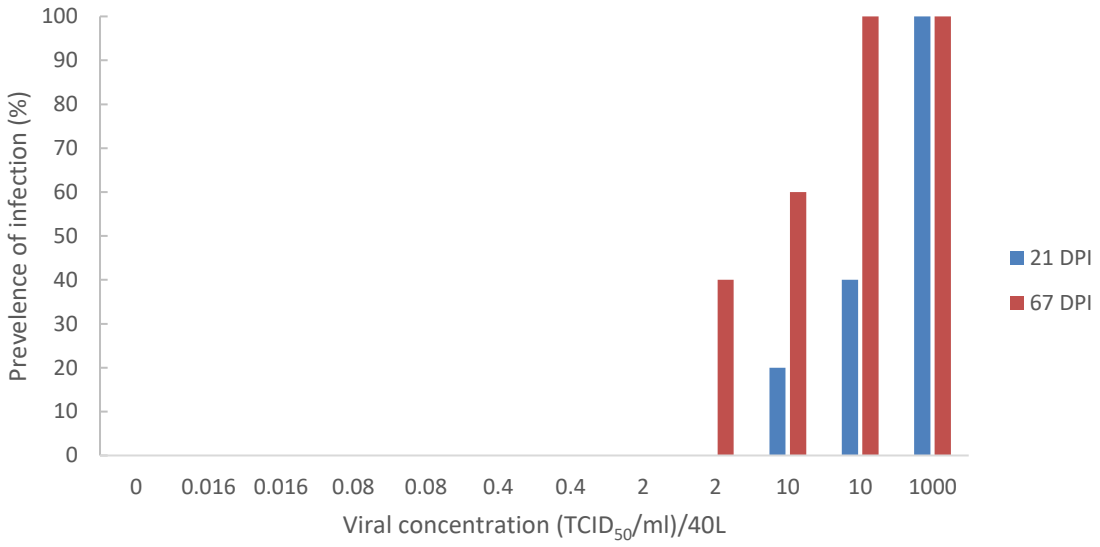



**Figure 3:** Prevalence of infection at 21 and 67 days post-infection (DPI) for determining the minimal infectious dose for ISAV in Atlantic salmon (*Salmo salar*). Fish were exposed to ISAV for 1 hour via bath challenge in duplicate 40 L tanks, with one additional bath receiving no virus (0, negative control) and another receiving a high dose (1000, positive control). Fish (n=5/tank) were sampled for analysis via RTq-PCR.

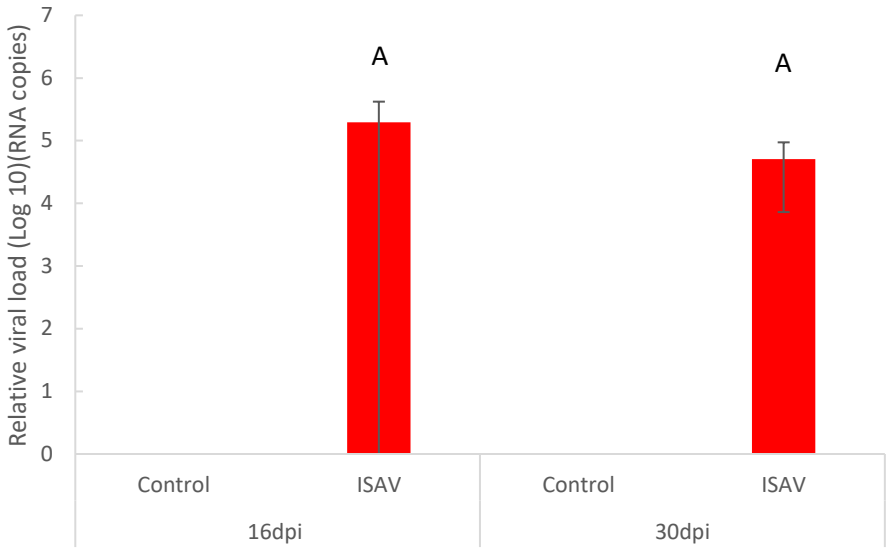



Figure 4: Relative viral loads, as determined by RT-qPCR in blood samples of sham-injected (0.1 mL sterile HBSS; control) and ISAV-injected (0.1 mL of $10^5 TCID_{50}$/mL) Atlantic salmon (*Salmon salar*) for respirometry analysis at 16 and 30 days post-infection (DPI) (mean ± standard deviation, n=5 fish/tank and 3 tanks/treatment) as determined by RT-qPCR in blood samples. Different capital letters denote significant difference ($p < 0.05$) by analysis of a t-test.

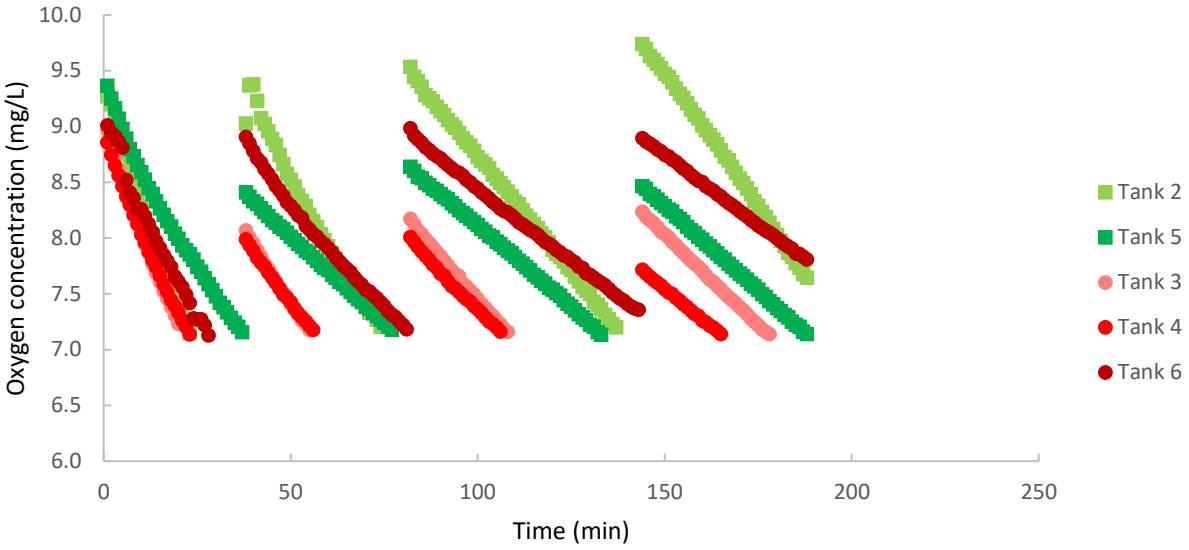



**Figure 5:** Tank oxygen concentrations during intermittent stop-flow respirometry at 16 days post-injection for sham-injected control (0.1 mL sterile HBSS, green) and ISAV-injected (0.1mL of $10^5$ $TCID_{50}$/mL, red) Atlantic salmon (*Salmo salar*). A "cycle" (as referred to in subsequent figures) consists of oxygen saturation levels brought up to 110%, followed by cessation of oxygen inflow, to measure fish oxygen consumption. Tank 1 had a malfunction; the optical probe was not 90° from the oxygen diffuser causing false readings and these values were excluded from analysis.

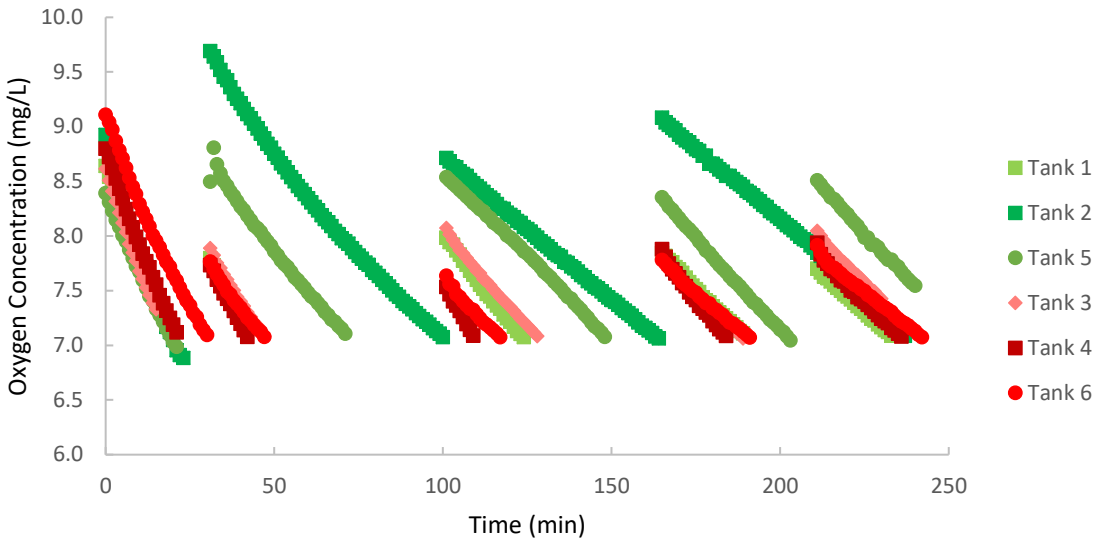



**Figure 6:** Tank oxygen concentrations during intermittent stop-flow respirometry at 30 days post-injection for sham-injected control (0.1 mL sterile HBSS, green) and ISAV-injected (0.1mL of $10^5$ $TCID_{50}$/mL, red) Atlantic salmon (*Salmo salar*). A "cycle" (as referred to in subsequent figures) consists of oxygen saturation levels brought up to 110%, followed by cessation of oxygen inflow, to measure fish oxygen consumption.

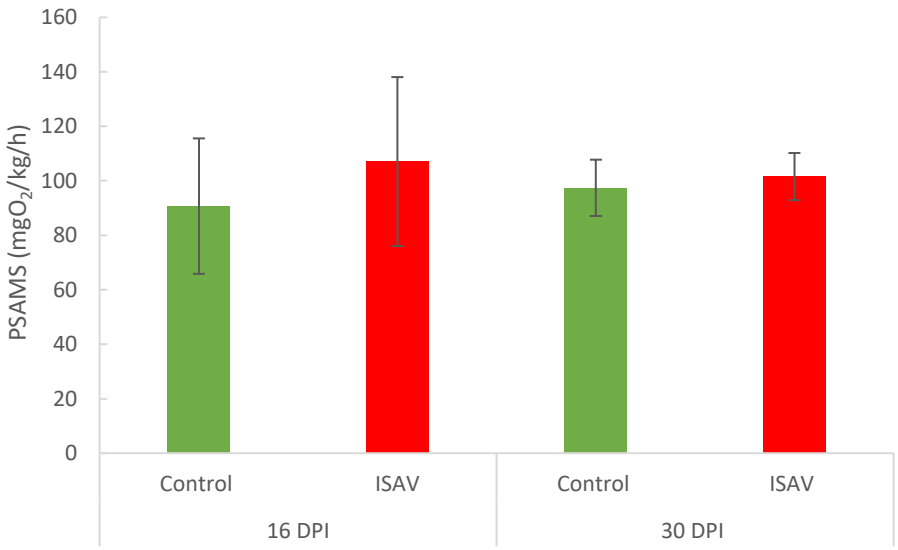



**Figure 7:** Post-stress metabolic aerobic scope (PSMAS) in Atlantic salmon (*Salmo salar*) at 16 and 30 days post-infection (DPI) with either 0.1 mL sterile HBSS (Control) or 0.1mL of $10^5$ $TCID_{50}$/mL of viral stock (ISAV) (mean ± standard deviation, n=3 tanks except for 16 DPI control where n=2 because tank 1 was excluded from analysis due to the optical probe being placed too close to the oxygen diffuser and giving false readings).PSMAS is the difference between the mean values of $MO_2$ PC1 and the mean values of $MO_2$ PRE. A two-way repeated measures ANOVA revealed no significant effects of treatment or sampling date.

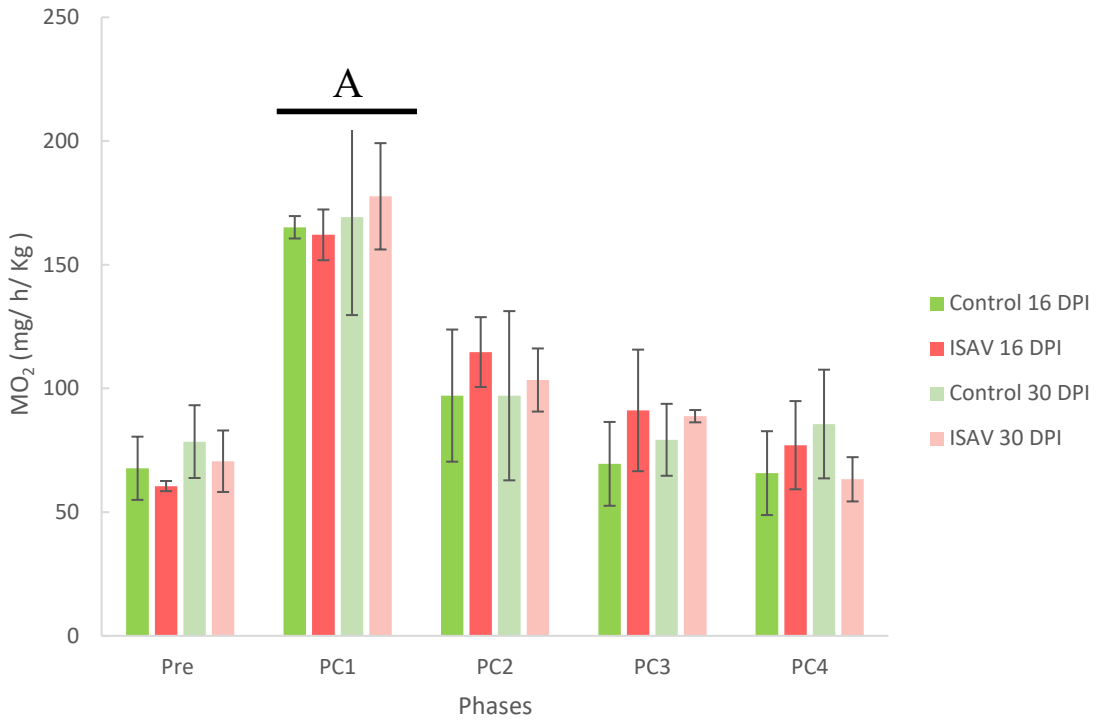



**Figure 8:** Oxygen consumption rate ($MO_2$) in Atlantic salmon (*Salmo salar*) at 16 and 30 days post-infection (DPI) with either 0.1 mL sterile HBSS (Control) or 0.1mL of $10^5$ $TCID_{50}$/mL of viral stock (ISAV) (mean ± standard deviation, n=3 tanks*). Pre: pre-chase stress measurements, PC1-4: sequential post-stress cycles. A cycle consists of oxygen saturation levels brought up to 110%, followed by cessation of oxygen inflow and measurement of fish oxygen consumption. All cycles have been aligned *post-priori* to have the same start time. *For tank 1 at 16 DPI only optical probe was not 90° from the oxygen diffuser causing false dissolved oxygen readings and values were excluded from analysis (16 DPI Control n=2). A two way repeated measures ANOVA revealed a significant effect in PC1 cycles ($p < 0.05$) denoted by A.

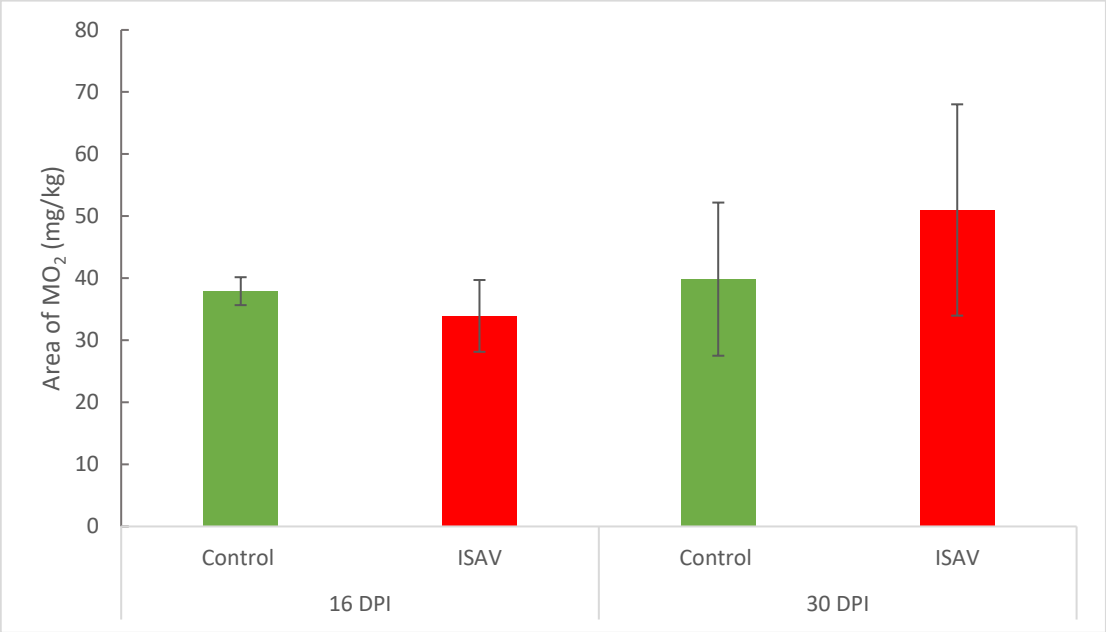



**Figure 9:** Excess post-stress oxygen consumption (EXPOC) in Atlantic salmon (*Salmo salar*) at 16 and 30 days post-infection (DPI) with either 0.1 mL sterile HBSS (Control) or 0.1mL of $10^5$ $TCID_{50}$/mL of viral stock (ISAV) (mean ± standard deviation, n=3 tanks except for 16 DPI control where n=2 because tank 1 was excluded from analysis due to the optical probe being placed too close to the oxygen diffuser and giving false readings). A two-way repeated measures ANOVA revealed no significant difference.

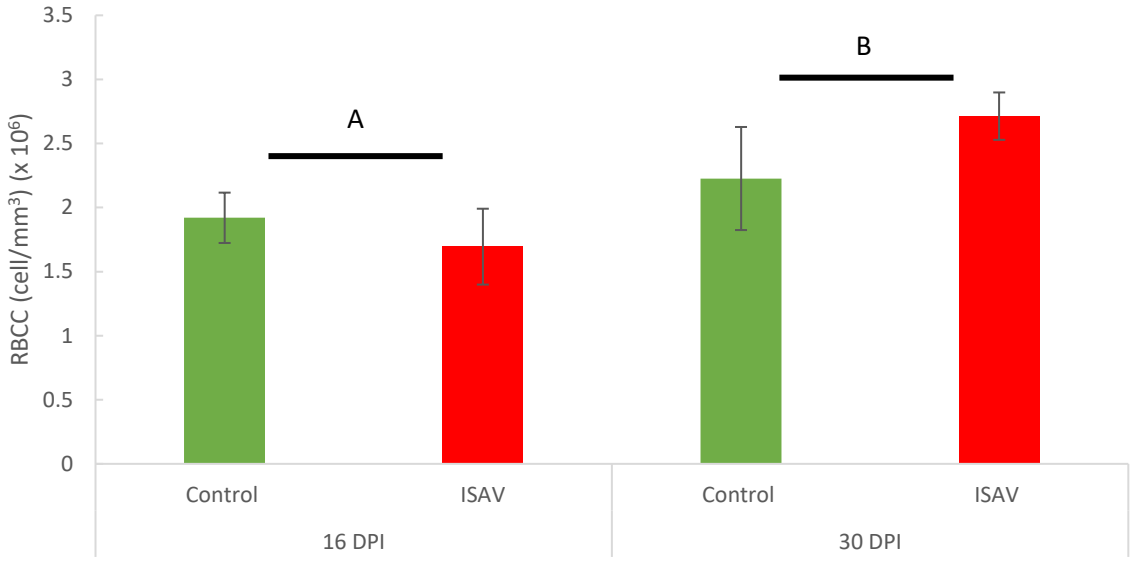



**Figure 10:** Atlantic salmon (*Salmo salar*) red blood cell count (RBCC) measured in the respirometry analysis at 16 and 30 days post-injection (DPI) with 0.1 mL sterile HBSS (control) or 0.1mL of $10^5$ $TCID_{50}$/mL of viral stock (ISAV) (mean ± standard deviation; n=5 fish per tank and 3 tanks per treatment). Different capital letters denote significant difference ($p < 0.05$) by *post-hoc* analysis of two-way ANOVA. Here 16 DPI groups were different than 30 DPI groups. Blood was collected just after completion of respirometry analysis.

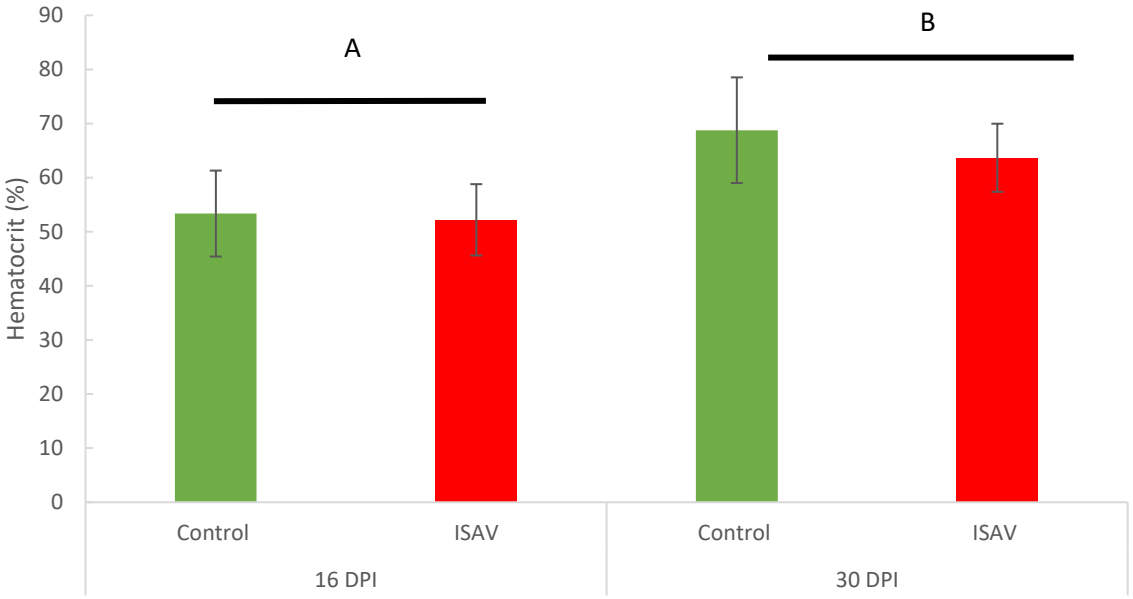



**Figure 11:** Atlantic salmon (*Salmo salar*) hematocrit measured in the respirometry analysis at 16 and 30 days post-injection (DPI) with 0.1 mL sterile HBSS (control) or 0.1mL of $10^5$ $TCID_{50}$/mL of viral stock (ISAV) (mean ± standard deviation; n=5 fish per tank and 3 tanks per treatment). Different capital letters denote significant difference ($p < 0.05$) by *post-hoc* analysis of two-way ANOVA. Here 16 DPI groups were different then 30 DPI groups. Blood was collected just after completion of respirometry analysis.

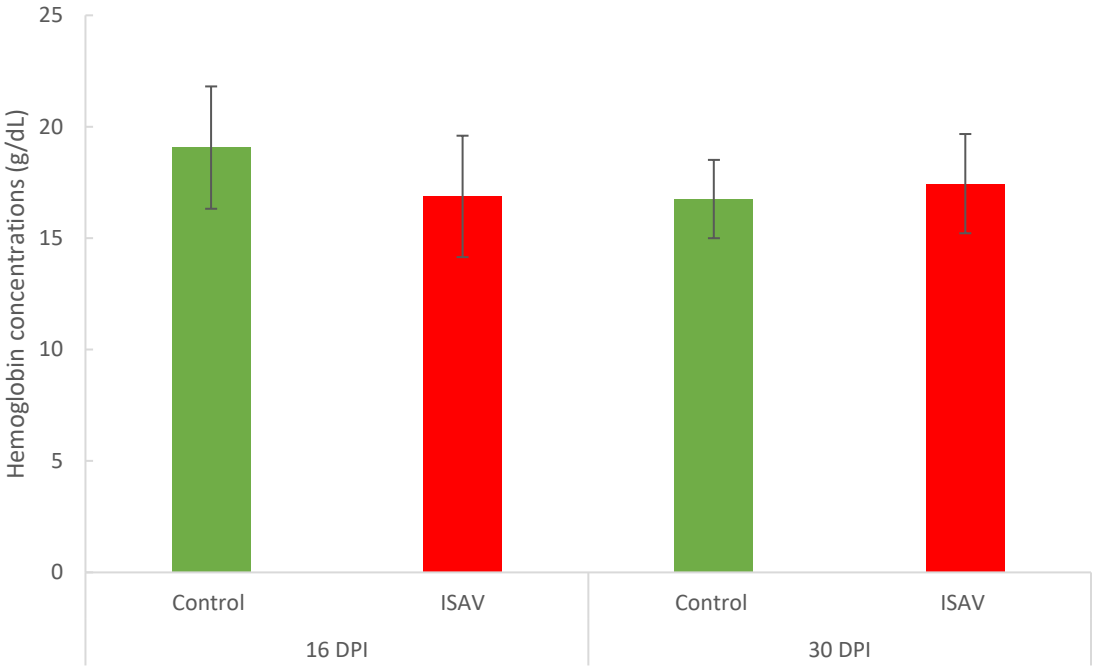



**Figure 12:** Atlantic salmon (*Salmo salar*) hemoglobin measured in the respirometry analysis at 16 and 30 days post-injection (DPI) with 0.1 mL sterile HBSS (control) or 0.1mL of $10^5$ $TCID_{50}$/mL of viral stock (ISAV) (mean ± standard deviation; n=5 fish per tank and 3 tanks per treatment). A two-way ANOVA revealed no significant effects of treatment or sampling date. Blood was collected just after completion of respirometry analysis.

**Table 3: Results of respirometry 2-way repeated measures ANOVA (PSAMS, $MO_2$ and EXPOC) 2-way ANOVA (RBCC, Hematocrit and Hemoglobin) testing the effects of date post-injection, status (sham- versus ISAV-injection) and interaction between date post-injection and status. Significant p-values are shown in bold.**

| Source of Variation | df | MS | F ratio | p-Value |
|---|---|---|---|---|
| PSAMS | | | | |
| Status | 1 | 436.9 | 3.906 | 0.143 |
| Date : Status | 1 | 104.2 | 0.931 | 0.405 |
| Residual | 3 | 111.9 | | |
| $MO_2$ | | | | |
| Status | 1 | 3.2 | 0.01 | 0.923 |
| Date : Status | 9 | 118.1 | 0.365 | 0.935 |
| Residual | 15 | 324.0 | | |
| EXPOC | | | | |
| Status | 1 | 52.68 | 0.386 | 0.554 |
| Date : Status | 1 | 152.54 | 1.117 | 0.326 |
| Residual | 7 | 136.53 | | |
| RBCC | | | | |
| Date | 1 | $6.58 \times 10^{12}$ | 81.75 | **<0.0001** |
| Status | 1 | $2.58 \times 10^{11}$ | 3.19 | 0.080 |
| Date : Status | 1 | $1.899 \times 10^{12}$ | 23.59 | **<0.0001** |
| Residual | 56 | $8.053 \times 10^{10}$ | | |
| Hematocrit | | | | |
| Date | 1 | 2708.16 | 44.84 | **<0.0001** |
| Status | 1 | 146.64 | 2.43 | 0.125 |
| Date : Status | 1 | 58.17 | 0.96 | 0.331 |
| Residual | 56 | 60.39 | | |
| Hemoglobin | | | | |
| Date | 1 | 0.11 | 5.01 | **0.029** |
| Status | 1 | 0.09 | 4.25 | **0.044** |
| Date : Status | 1 | 0.01 | 0.66 | 0.421 |
| Residual | 56 | 0.02 | | |

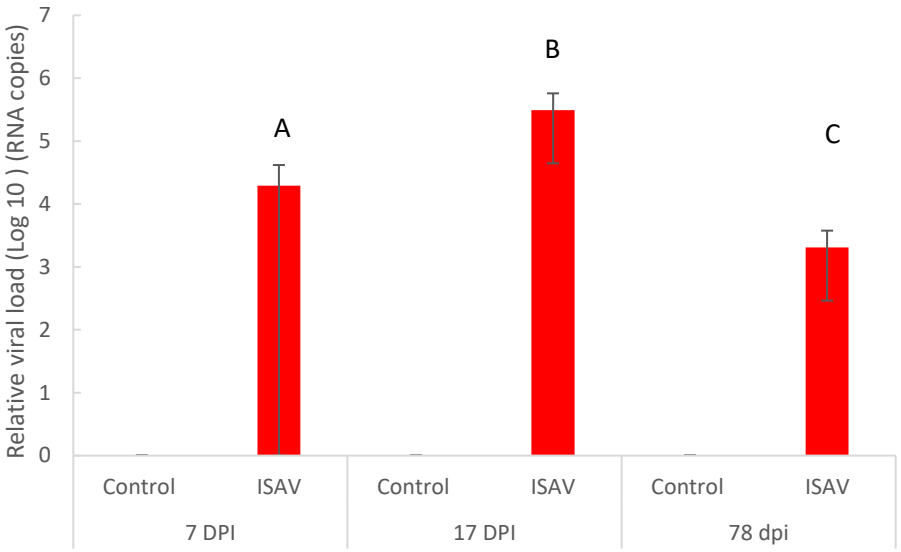



Figure 13: Relative viral loads, as determined by RT-qPCR in blood samples of sham-injected (0.1 mL sterile HBSS; control) and ISAV-injected (0.1 mL of $10^5$ $TCID_{50}$/mL) Atlantic salmon (*Salmo salar*) for protein synthesis analysis at 7, 17 and 78 days post-infection (DPI) (mean ± standard deviation, n=5 fish/tank and 3 tanks/treatment) as determined by RT-qPCR in blood samples. Different capital letters denote significant difference ($p < 0.05$) by post-hoc analysis of one-way ANOVA.

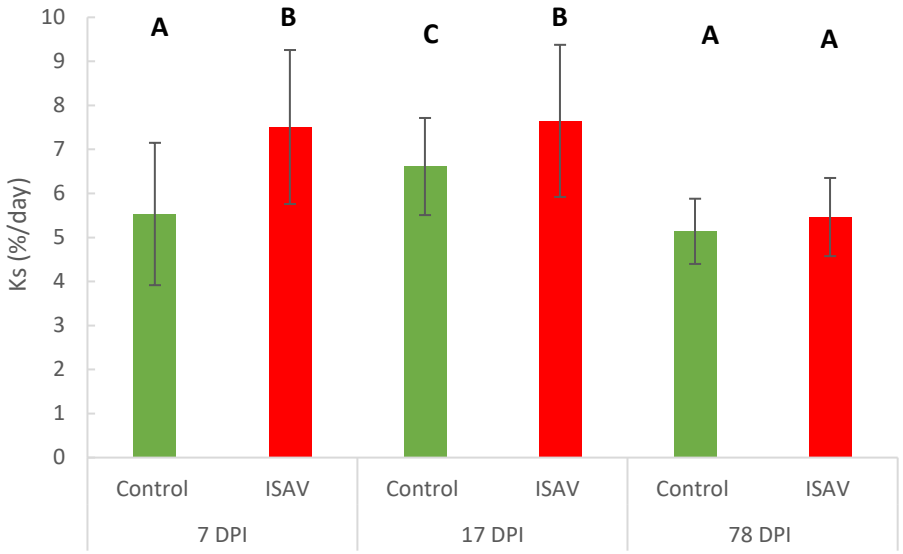



**Figure 14:** Protein synthesis rates (Ks) in Atlantic salmon (*Salmo salar*) at 7, 17 and 78 days post-infection (DPI) with either 0.1 mL sterile HBSS (Control) or 0.1mL of $10^5$ $TCID_{50}$/mL of viral stock (ISAV) (mean ± standard deviation, n=3 tanks with 5 fish/tank). (mean ± standard deviation, n=3 tanks). Different capital letters denote significant difference ($p < 0.05$) by *post-hoc* analysis of two-way ANOVA.

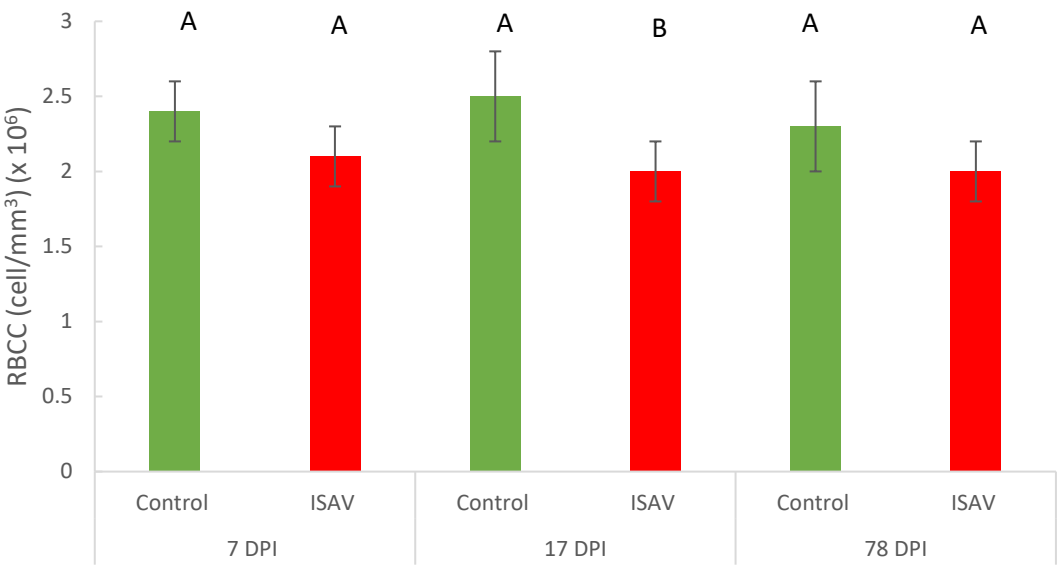



**Figure 15:** Atlantic salmon (*Salmo salar*) Red Blood Cell Counts (RBCC) (cells/mm$^3$) (mean ± standard deviation) of the fish used in the Protein Synthesis analysis. Control fish (n=5/tank 3 tank replicates) and ISAV + (n=5/tank 3tank replicates) were sampled at three-time points measured in Days Post Infection (DPI) 7DPI, 17DPI, and 78DPI. Following a Two-way ANOVA, A not significantly different (p-value >0.05) from A but significantly different from B.

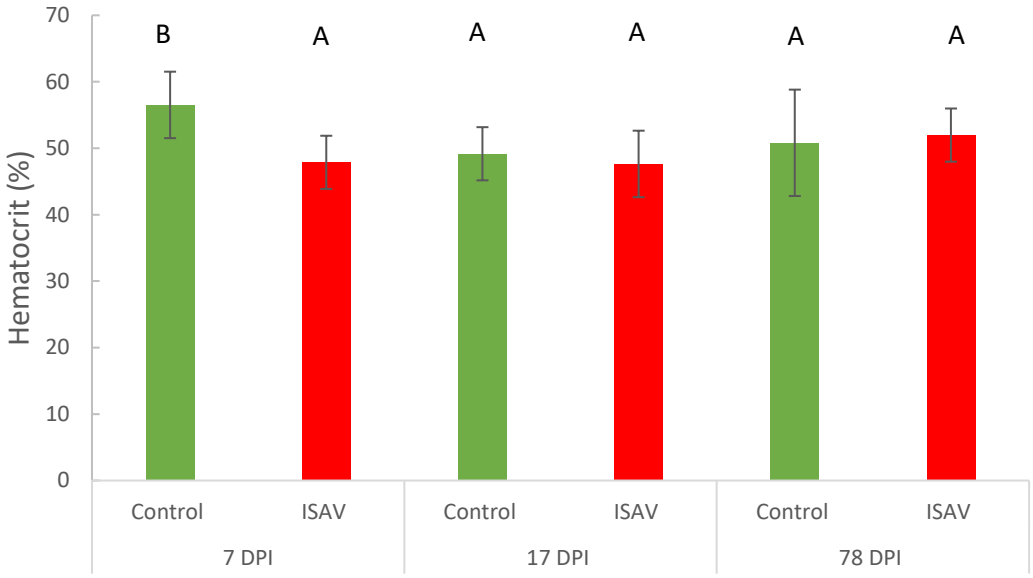



**Figure 16:** Atlantic salmon (*Salmo salar*) hematocrit percentages (%) (mean ± standard deviation) of the fish used in the Protein Synthesis analysis. Control fish (n=5/tank 3 tank replicates) and ISAV + (n=5/tank 3tank replicates) were sampled at three time points measured in Days Post Infection (DPI) 7DPI, 17DPI, and 78DPI. Following a Two-way ANOVA, A significantly different from B (p-value ≤ 0.05).

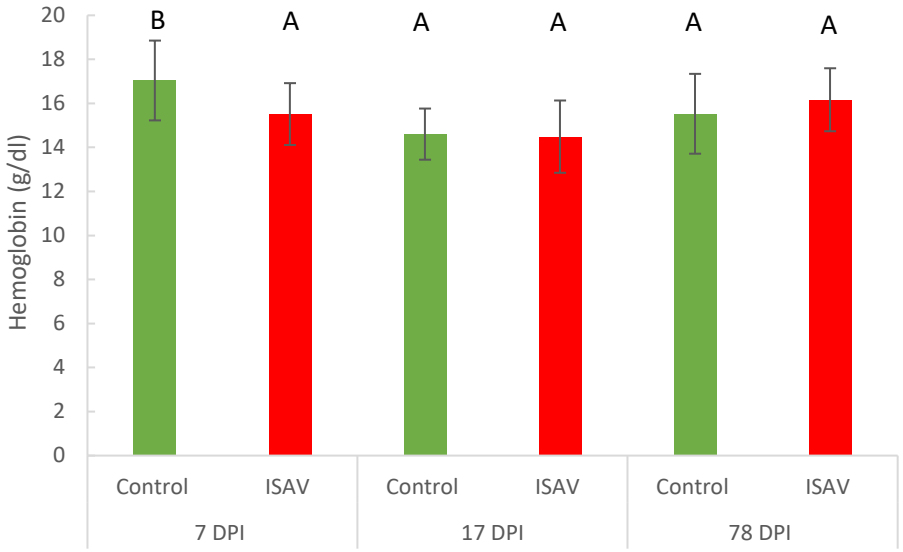



**Figure 17:** Atlantic salmon (*Salmo salar*) hemoglobin concentration (g/dl) (mean ± standard deviation) of the fish used in the Protein Synthesis analysis. Samples screened at a wavelength of 540nm. Control fish (n=5/tank 3 tank replicates) and ISAV + (n=5/tank 3tank replicates) were sampled at three time points measured in Days Post Infection (DPI) 7DPI, 17DPI, and 78DPI. Following a Two-way ANOVA, A significantly different from B (p-value ≤ 0.05).

**Table 4: Results of 2 way ANOVA models for protein synthesis testing the effects of the date post injection, treatment and interactions between date post injection and status of infection. Significant p-values are shown in bold. *CT value was analyzed with a one-way ANOVA.**

| Source of Variation | df | MS | F ratio | p-Value |
|---|---|---|---|---|
| *CT Value | | | | |
| Date | 2 | 7.50 | 34.35 | **<0.0001** |
| Residual | 42 | 0.20 | | |
| | | | | |
| Protein Synthesis Rate | | | | |
| Date | 2 | 19.51 | 12.71 | **<0.0001** |
| Status | 1 | 21.07 | 13.73 | **0.0004** |
| Date : Status | 2 | 4.87 | 3.17 | **0.048** |
| Residual | 77 | 1.53 | | |
| | | | | |
| RBCC | | | | |
| Date | 2 | $8.055 \times 10^{10}$ | 1.35 | 0.265 |
| Status | 1 | $2.401 \times 10^{12}$ | 40.19 | **<0.0001** |
| Date : Status | 2 | $2.099 \times 10^{11}$ | 3.51 | **0.0342** |
| Residual | 84 | $5.974 \times 10^{10}$ | | |
| | | | | |
| Hematocrit | | | | |
| Date | 2 | 0.01 | 4.84 | **0.010** |
| Status | 1 | 0.02 | 8.39 | **0.005** |
| Date : Status | 2 | 0.02 | 7.86 | **0.001** |
| Residual | 84 | 0.03 | | |
| | | | | |
| Hemoglobin | | | | |
| Date | 2 | 24.37 | 9.99 | **0.0001** |
| Status | 1 | 2.51 | 1.03 | 0.313 |
| Date : Status | 2 | 9.06 | 3.71 | **0.029** |
| Residual | 84 | 2.44 | | |

**Table 5: Results from Pearson's correlation coefficient test between relative viral load and various blood parameters on pooled data from fish used in respirometry (n=58) and fish used for the protein synthesis analysis (n=88). The cutoff value for significance was set at 0.05. Significant p-values are in bold.**

| Blood parameter | Pearson's R | p-value |
|---|---|---|
| RBCC (Respirometry) | 0.082647 | 0.5301 |
| Hematocrit (Respirometry) | -0.19395 | 0.1376 |
| Hemoglobin (Respirometry) | -0.17355 | 0.1848 |
| RBCC (Protein) | -0.50516 | **$3.8 \times 10^{-7}$** |
| Hematocrit (Protein) | -0.3054 | **0.003423** |
| Hemoglobin (Protein) | -0.21591 | **0.04097** |
| Ks Ratio (Protein) | 0.44851 | **$9.28 \times 10^{-6}$** |

# CHAPTER 4 – DISCUSSION AND CONCLUSION

This study aimed to determine if wild-type salmon infected with ISAV at sub-lethal levels would incur a higher metabolic cost than sham-injected controls after a period of acute stress. Other pathogens, such as the amoeba *Paramoeba perurans* and the bacteria *Tenacibaculum maritimum*, affect the metabolic rate of fish by directly influencing the rate at which they take up oxygen, e.g., through gill damage (Leef *et al*. 2005, Jones *et al*. 2007, Seppanen *et al*. 2009, Hvas *et al*. 2017). A rapid recovery rate in wild fish is essential to their survival (Lee *et al.* 2003b, Zhang *et al.* 2018) as this can affect their capacity to evade predators and activities such as migration. To quantify the costs of ISAV infection, respirometry was used to determine $MO_2$ (i.e., metabolic cost) before and after acute chasing stress, as well as various blood parameters. Additionally, the rate of protein synthesis in the kidney was measured in a separate group of infected and non-infected fish at various stages of infection. It is important to note here that fish were considered recovered in the respirometry analysis when $MO_2$ values had returned to pre-stress RMR values. Fish cannot be classified as genuinely recovered as primary (plasma cortisol) indicators, and secondary stress (osmoregulation) response was not measured.

**Minimal Infectious Dose challenge and chosen viral concentration**

The MID determined for this study was 10 $TCID_{50}$/mL. However, this range did not generate 100 % prevalence of infection until 67 DPI. The only tank with 100 % prevalence at both sample dates (21 and 67 DPI) was the positive control tank ($10^3$ $TCID_{50}$/mL). Compared to previous experiments using a more virulent strain of ISAV,

the viral strain chosen had a low virulence (D. Ditlecadet, personal comm. DFO 2019). When using IP injection with ISAV, Trojan fish had 100 % prevalence of infection, but they had not infected naïve cohabitating fish after seven days. This is not usually the case with ISAV infections, although reduced infectivity was observed on at least one previous occasion (Leblanc *et al*. 2012). This can be due to the strain itself or be caused by a difference in resistance in the wild salmon used for the experiment. It is also possible that the time elapsed since smoltification was longer and so the fish had recovered from this stress and were therefore less susceptible to infection. Seven days may have been too short for the cohabitation infection to take hold for this viral strain. To ensure that a relatively uniform infection status was achieved before respirometry experiments, all fish were IP-injected with the virus. Natural exposure obtained by cohabitation mimics the ISAV infection pathway, but in past experiments (LeBlanc *et al*. 2018), the ISAV symptoms observed during injection challenges were consistent with natural ISAV infections.

*Viral load*

HPR-deleted ISAV strains have the potential to be very virulent, as is the case with HPR4. ISAV Strain NS/2012-21 of East Coast Canada (Nova Scotia) has been observed to cause 27-71 % mortality in IP-injected (0.1mL @ $10^5$ $TCID_{50}$/mL) commercially reared Atlantic salmon used in a cohabitation model. When comparing HPR4 and NS/2012-21 the latter had a mid-range level of virulence. Leblanc *et al.* (2018) saw a peak of relative viral load at 28 DPI with a mean value of approximately $10^6$. This same

viral dose of NS2012-21 was IP injected into fish for the respirometry and the protein synthesis analysis in the present study.

For both the respirometry and protein synthesis analyses, relative viral loads followed a similar trend over time. Looking at both analyses in chronological order, peak relative viral loads ($10^5$ RNA copies) occurred at 17 DPI (protein synthesis analysis), followed by a downward trend by 10-fold at 30 DPI (respirometry analysis) and a further 10-fold at 78 DPI (protein synthesis analysis). Using the same initial dose of virus for IP injection, the results here suggest that there may be an overall lower viral load and viremia in wild-type fish (i.e., $10^5$ RNA copies) compared to farmed salmon ($10^6$ RNA copies; LeBlanc *et al.* 2018), meriting further investigation. It cannot be ruled out that the 16 DPI sample date may have been past the peak infection.

The respirometry trial viral loads ($10^{5.29}$ RNA copies at 16 DPI and $10^{4.70}$ RNA copies at 30 DPI) did not induce anemia in the injected fish. The only fish with a measurable anemic response were those at 7 DPI and 17 DPI in the protein synthesis experiment, with a viral load of $10^{4.2929}$ RNA copies and $10^{5.49}$ RNA copies respectively (see Appendix Table 3).). It is important to note that relative viral loads below $10^{3.31}$ RNA copies did not induce anemia in hosts at 78 DPI in the protein synthesis trial. These observations raise an interesting question about how different relative viral loads can affect the physiology of the host. One such question is why the fish were anemic at 7 DPI in advance of the peak infection, which could be the subject of future research.This suggests that there may be a minimal quantity of ISAV that the Tobique River wild-type salmon can live with without affecting their physiology. Compared to LeBlanc *et al.*

(2018), relative viral loads of $10^6$ RNA copies were observed to cause mortality in farmed salmon.

IP injection rather than cohabitation (i.e., as done by LeBlanc *et al.* 2018) was chosen because wild Trojans could not shed enough virus to cause infection on the naïve cohabitants in a measurable manner after seven days. This is an interesting observation, especially considering that wild fish are not likely to remain in contact with infected farmed fish for so long. Bath challenges and cohabitation models are more representative of natural routes of infection even though they produce lower relative viral loads (Aoki *et al.* 2005, LeBlanc *et al.* 2018, Adams 2019, Munang'andu and Evensen 2019). IP injections at time zero of each experiment ensure a similar and synchronised exposure for each fish but are less representative of natural infections as the pathogen has bypassed some of the host's immune defenses.

Here, the continued detection of ISAV at 78 DPI suggests that viral replication, even though significantly reduced from 17 DPI, could maintain a viral population within the host fish.

**Impact of ISAV Infection on Metabolic Response to Acute Stress (Respirometry analysis)**

It is important to note that no anemia was observed in the ISAV+ fish in the samples taken. In contrast, anemia was observed in ISAV+ fish in the protein synthesis experiment at 7 DPI via hemoglobin and hematocrit and 17 DPI via RBCC only. This suggests that limited conclusions can be drawn from these sets of challenges as the comparison is no longer ISAV+ anemic fish versus control non-anemic fish for

respirometry. The comparison is now with sub-lethally infected fish with no anemia versus control fish with no anemia. The original objective of the respirometry experiment was not met, and ideally, these challenges would be repeated with ISAV+ anemic fish. However, this is evidence that the infection does not have to result in anemia if the infection is sub-lethal. Other ISAV symptoms were not observed or measured but cannot be excluded. As outlined above, other pathogens (*P. perurans* and *T.maritimum*) have affected the MR of their hosts without causing anemia. In our study, no measurable effect on the metabolic rate was detected. During the protein synthesis trial, low levels of anemia were observed at 7 and 17 DPI, and protein synthesis rate was increased in these fish. Possible explanations for this could be due to erythropoiesis and the mounting of of an immune response. The protein synthesis results indicate that even with low levels of anemia, sub-lethal effects are transpiring in the infected fish. It has been observed that there is augmented activity (gene up- and down-regulation and generation of new blood cells, i.e., erythropoiesis) in the head kidney of salmon with acute stress associated with ISAV infection (Leblanc *et al.* 2010, Leblanc *et al.* 2012, Krasnov *et al.* 2013).

There are a few possible explanations for this outcome that are worth elaborating on. The first is that the fish used in this study were larger than the post-smolt size used in other many ISAV studies. Fish have been observed to be more susceptible to ISAV during smoltification due to the stress of this process (Glover *et al*. 2006); this size difference may reflect that fish had regenerated the energy stores lost during smoltification, and this may have influenced their immune response. Second, wild-type fish may have some natural resistance to this strain of the virus that is reduced in farmed fish. This would merit further investigation. No farmed salmon controls were raised in

the same conditions as the wild-type fish for comparison used in this study, so this conclusion cannot be affirmed presently. Anemia is often a clinical symptom of ISAV, however, not a guarantee (CFIA 2020 b). The sub-lethal dose of the virus was certainly not able to generate any level of detectable anemia in the respirometry fish. Blood was sampled after the respirometer analysis was completed not to stress the fish more than they would be from the handling. Even though the respirometry fish did not develop anemia, the ISAV+ fish still had a high viral load, meaning that a sub-lethal infection was still achieved.

Sub-lethal ISAV infection did not affect the aerobic response/recovery ($MO_2$, PSAMS or EXPOC) to acute stress in wild salmon when using a group respirometry model. Studies outlining the respirometry technique for measuring oxygen uptake (e.g., Chabot *et al*. (2016) and describing intermittent stop-flow respirometry (e.g., Svendsen *et al*. (2016) typically focus on individual fish. These techniques were modified to estimate the cost of a sub-lethal ISAV infection at the tank level using groups of fish. ). This rapid recovery comes with a need for higher $MO_2$ (Fivelstad and Smith 1991, Forsberg 1994).

Zhang *et al*. (2019) tested domesticated Atlantic salmon (Mowi-McConnell strain) of 75g at 11 ºC with a different virus (piscine orthoreovirus). They found no difference between control and infected fish, approximately 175 $mgO_2$/kg/h for RMR and approximately 375 $mgO_2$/kg/h for maximum oxygen consumption (fish were chased for 7 min). Comparing Zhang *et al.* (2019) values to those of the present respirometry analysis of wild type salmon of 241 ± 10 g at 12 ºC RMR (60-80 $mgO_2$/kg/h) and maximum oxygen consumption (between 162 ± 10 $mgO_2$/kg/h and 178 ± 21 $mgO_2$/kg/h),aa similar recovery pattern in the fish was observed.

One study from Farrell (2006) looked at the $MO_2$ values of a large (n > 10 000 fish) group of farmed Atlantic salmon on a transport vessel at 10 – 13 ºC (density approx. 100 $kg/m^3$ with an average fish mass of 5.66 kg). This large group of fish at high density and submitted to the stress of transportation consumed 480-240 $mgO_2/kg/h$ for over four hours. Farrell (2006) concluded that these bulk $MO_2$ values were comparable to values of single fish measured in laboratory settings (between 2.2 and 4.5 $mgO_2/kg/min$, Farrell *et al* 2003, Lee *et al.* 2003), indicating that stress augments $MO_2$ values. Comparing Farrell bulk $MO_2$ values to the ISAV respirometry data, it is possible to see that the values we obtained are within a relevant range.

The EXPOC values obtained in this present study ranged from 3333.92 ± 5.79 mg $O_2$/kg to 5050.99 ± 17.02 mg $O_2$/kg for control and ISAV+ wild-type Atlantic salmon, respectively, at 12 ºC. This range is lower than values found in the literature. For instance, Zhang *et al.* (2016) reported EPOC values for wild Atlantic salmon (body mass = 32 ± 0.5 g) to be 914 ± 55.7 mg $O_2$/kg at 12 ºC. However, they chased their fish to exhaustion, taking approximately 20 min, whereas the chasing protocol used in the current study was only 2 min and the fish were not fully exhausted. The difference between EPOC and EXPOC is that the former considers actual RMR, and measures are taken on individual fish. It is of note that the majority of studies have been completed on fish of smaller size closer to the post-smoltification stage. The wild fish here followed the same phases of recovery as outlined by Zhang *et al* (2018). The fast recovery phase followed the same duration as Zhang had observed (see Introduction above).

**Impact on the rates of protein synthesis**

Infection with a sub-lethal ISAV dose had other physiological costs in this study, as demonstrated by the significantly elevated protein synthesis rate in the kidney of ISAV+ fish at 7DPI and 17 DPI (35.8 and 15.7 % higher than controls, respectively). RBCC were 10% lower in ISAV fish at 17 DPI, and hemoglobin values at 7 DPI were 7 % lower in ISAV+ fish, compared to controls. Higher values of protein synthesis rates for the 17 DPI control fish were unexpected, but could be due to fish growth.

Protein synthesis is known to increase in response to viral infections (Workenhe et al. 2007, Garcia-Rosado et al. 2008, Das *et al.* 2007, LeBlanc *et al*. 2012, Aamelfot *et al.* 2016) as fish build an immune response (Magnadóttir 2006, Leblanc *et al.* 2010, LeBlanc et al. 2012, Collet *et al.* 2015). Increased protein synthesis is required to maintain elevated erythropoiesis triggered by anemia (Krasnov *et al.* 2013). Studies have shown that many genes associated with immune response pathways are regulated in response to ISAV infection, such as the CC-chemokine 19, the interferon-induced GTP-binding protein matrix MX and the blood complement factor C5 (LeBlanc *et al*. 2010, Leblanc *et al.* 2012, Garcia *et al*. 2013 and Johansen *et al*. 2019). This is one possible explanation for the increased protein synthesis rates in ISAV+ fish at 7 and 17DPI.

High relative viral loads of $10^{3.08}$ RNA copies in the ISAV + fish used for protein synthesis analysis suggest that the virus was still replicating within the host at 78 DPI. This is the first study to observe how protein synthesis rates in wild salmon are affected by ISAV infection. The ISAV + fish protein synthesis rates were not different from the control group at 78 DPI. Fish still presented a significant viral load at 17 and 78 DPI. These results suggest that the ISAV+ fish may be allocating resources to replenish the

cells that ISAV infects, i.e., endothelial and blood cells (Weli *et al.* 2013 and Aamelfot *et al.* 2014).

*Blood analysis in all challenges*

When comparing the hematological results of the respirometry and protein synthesis analyses, there are some differences. In the respirometry analysis, no detectible anemia was found despite fish having a high viral load confirming infection. Advanced-stage ISAV infections have been observed to cause severe anemia in host populations, but this is not a definitive outcome (Evensen *et al.* 1991, Rimstad *et al.* 1999, Aamelfot *et al.* 2014, Rimstad *et al.* 2019, CFIA 2020 b). Relative viral loads below $10^5$ RNA copies were likely insufficient to overwhelm the host immune response; further investigation into this subject would be needed.

Changes in stocking density have been observed to affect hemoglobin levels in Atlantic salmon fry, increasing when densities are high (above 25 $kg/m^3$) and decreasing when densities are low (below 25 $kg/m^3$) (Hammenstig *et al.* 2014).Fish density in the current study varied from 10.8 to 27.4 $kg/m^3$ due to movement between tanks, and this could have affected their hemoglobin levels.. It is of note that all the blood parameters of these wild-type salmon (both controls and ISAV+ fish) were higher than those found in farmed salmon (Sandnes and Waagbø 1988). This could also be due to the natural variance in the wild salmon coupled with the effects of stress. The blood parameters were equally elevated in both respirometry and protein synthesis trials, suggesting that the fish might have a naturally higher range. Perhaps the fish did not have a long enough time to adjust to the new stocking densities. Iversen *et al*. (1998) observed that if salmon smolts

did not have enough time to recover between stressor events, this would reduce their ability to handle multiple stressors.

The wild fish could potentially be experiencing greater stress in captivity during the challenges as compared to in the wild stress affecting them in their natural environment (Barton and Iwama 1991, Carey and McCormick 1998, Murray *et al.* 2017). There is also a genetic variation factor that could come into play. Breeding information is lacking for these fish and all that was known is they are the F1 generation of wild parents (90 males with 90 females). In future studies attention to the genetic background would be helpful.

There were no correlations between relative viral loads and blood parameters in the respirometry analysis. A significant negative correlation with relative viral load was observed with the blood parameters (RBCC, hematocrit, and hemoglobin) in the protein synthesis analysis.

**Parallels between Avian Influenza A and NS/2012 infections in wild host exploitation:**

Avian influenza A, another member of the Orthomyxoviridae family, is thought to be well adapted to its wild-type bird hosts (Webster 2002). Wild birds exhibit only mild symptoms when infected with an influenza strain (H5N1). In contrast, domesticated birds seem to be maladapted to this strain, suffering a much higher physiological impact (Nelson and Holmes 2007, Chen *et al.* 2004). It is possible to draw parallels between these observations and the data collected in this thesis. Only wild-type salmon were observed here; they did show apparent signs of infection but had measurable effects on

blood parameters and protein synthesis at the peak of infection in one of two infection groups, with no physiological impact on their aerobic scope. This supports the hypothesis that ISAV may be attenuated in wild-type salmon with more of a commensalism type relationship. However, further investigation is needed to make a more concrete conclusion.

## Conclusion

Wild salmon infected sub-lethally with ISAV NS/2012 in this study showed overall a minimal physiological cost. In this study, they were more robust to an IP-injected dose of NS/2012-12 virus that proved lethal to commercially reared stocks (Leblanc *et al.* 2018) as no mortalities were observed here. Some parameters within the fish infected for the protein synthesis experiment were significantly affected as expected with anemia; however, no clinical signs of anemia were observed in the fish infected for the respirometry experiments. This was an important factor in the respirometry trials without anemia in the ISAV+ fish; there are limited conclusions that can be made. However, fish remained healthy, leading to the conclusion that the fish's innate and acquired immune response was active and prevented the virus from causing apparent signs of morbidity. During acute stress periods, all salmon showed a significant increase in $O_2$ uptake in the PC1 (max $MO_2$). Further, all fish showed recovery patterns similar to those found in the literature (Zhang *et al.*2016). However, there did seem to be differences between blood parameters for farmed salmon found in literature and the values obtained here for wild salmon. This merits further investigation. Knowledge of genetic variability would also be important to document.

In this experiment, the objective was to determine if sub-lethal ISAV could affect wild salmon's performance and physiology. The relative viral loads associated with infection in wild-type fish did not cause anemia in the respiration challenge and did not impair aerobic response to acute chasing stress. The results found in these analyses suggest that a sub-lethal infection with ISAV strain NS2012-21 has a minimal physiological cost in wild-type Atlantic salmon. ISAV strains have caused outbreaks of variable virulence in farmed salmon populations (Gagné and Leblanc 2017). This choice of viral strain is relevant for the situation in the Atlantic Canadian region as it represents one of the strains of current concern in this area (LeBlanc *et al.* 2018).

The level of virulence of a particular HPRΔ strain influences the speed to reach a systemic infection in the host (McBeath *et al*. 2014). A sub-lethal dose was important as a higher dosage may have caused mortality in the fish after the acute stress in the respirometry method. It would not have been possible to observe the effects of infection. ISAV's entry mechanism into the host may vary with strain and must bypass the mucosal surfaces (Aamelfot *et al.* 2015). The use of IP injection was a way to ensure a uniform infection in all fish. Wild fish would not receive such an acute dosage of ISAV at a single exposure time. Instead, they would be exposed to gradual concentrations depending on various factors, including viral shedding rates from farm sites, wild fish population densities, and environmental conditions.

Wild salmon can be carriers of ISAV (Nylund *et al.* 2003, Rimstad *et al.* 2011). The exposed fish received an amount of virus that was lethal to commercially reared fish, although they only developed a sub-lethal infection in this study. Genetic variation of commercial and wild-type Atlantic salmon could contribute to the virulence level that a

viral strain has. Other considerations include viral mutation during cell passage and that the fish were no longer under the recovery of post smoltification stress. The data of this thesis would suggest that ISAV NS2012-21 would be attenuated in the wild-type salmon used.

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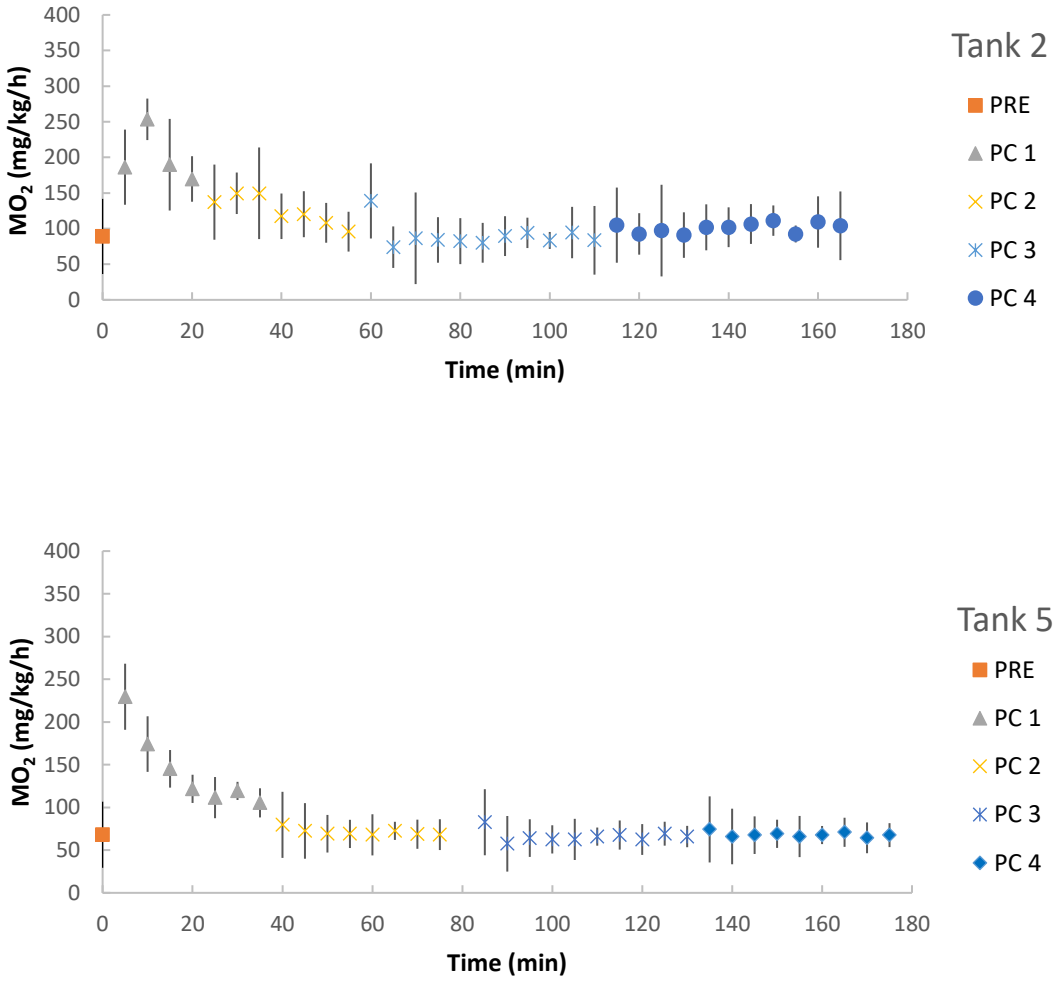
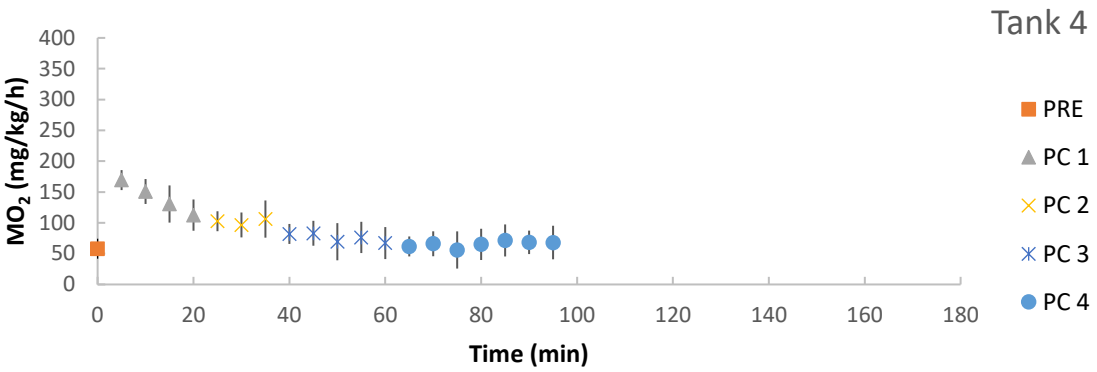
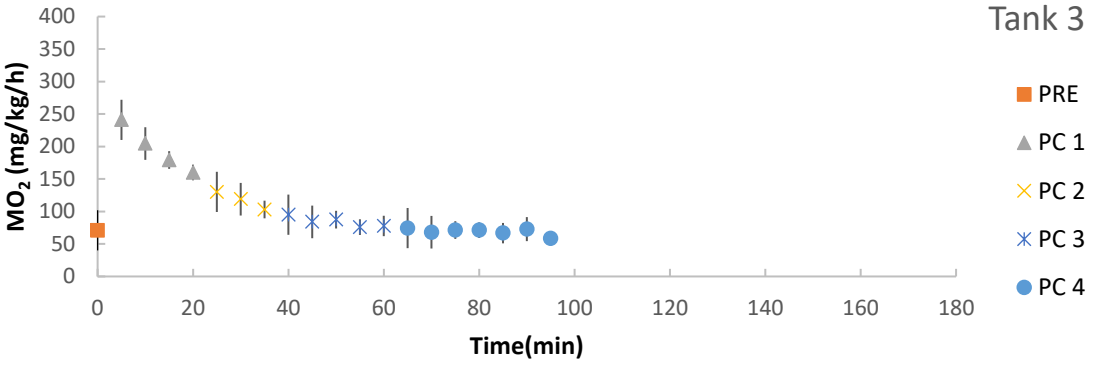
# Appendix:

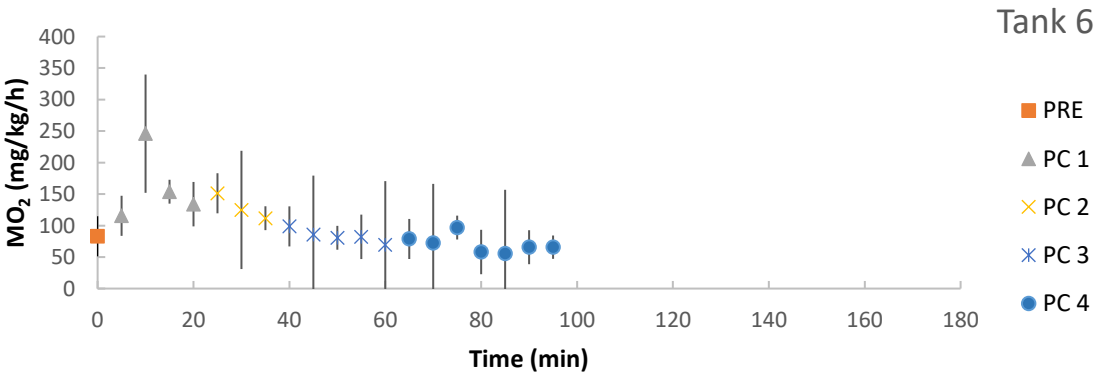
Appendix figures 1 through 4 are the graphical representations of the metabolic rates of group fish in tanks. The PRE value is the approximation of RMR value used to establish pre-chasing stress. PC 1 through PC 4 are the post-chasing stress metabolic rates. Figures 5 and 6 are the sections of the respirometry analysis that were used to calculate the EXPOC values by integrating the area under the curve.



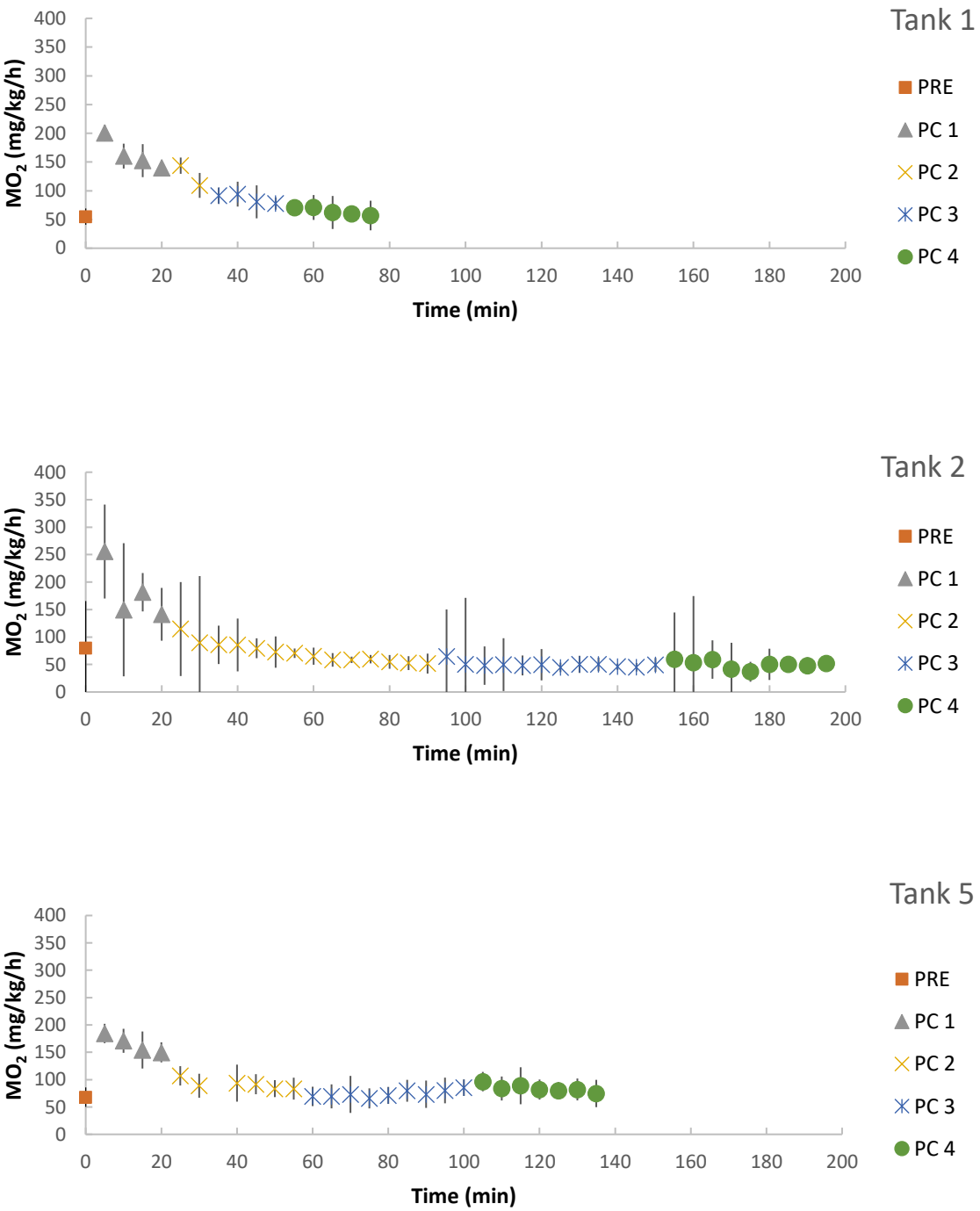

**Appendix Figure 1:** Atlantic salmon (*Salmo salar*) $MO_2$ values for individual control tanks at 16 days post-infection. Each point is the mean ± standard deviations of 5 values collected at 1 min intervals. Colours identify the different cycles measured in the respirometry analysis (PRE = pre-stress, PC1-4 = sequential post-stress cycles). EXPOC is represented by the area under this curve defined by the first point immediately after the PRE value to the first $MO_2$ to come within 5% of the PRE value.


Tank 4
MO2 (mg/kg/h)
400
350
300
250
200
150
100
50
0
0
20
40
60
80
100
120
140
160
180
Time (min)
PRE
PC 1
PC 2
PC 3
PC 4


Tank 3
MO2 (mg/kg/h)
400
350
300
250
200
150
100
50
0
0
20
40
60
80
100
120
140
160
180
Time(min)
PRE
PC 1
PC 2
PC 3
PC 4

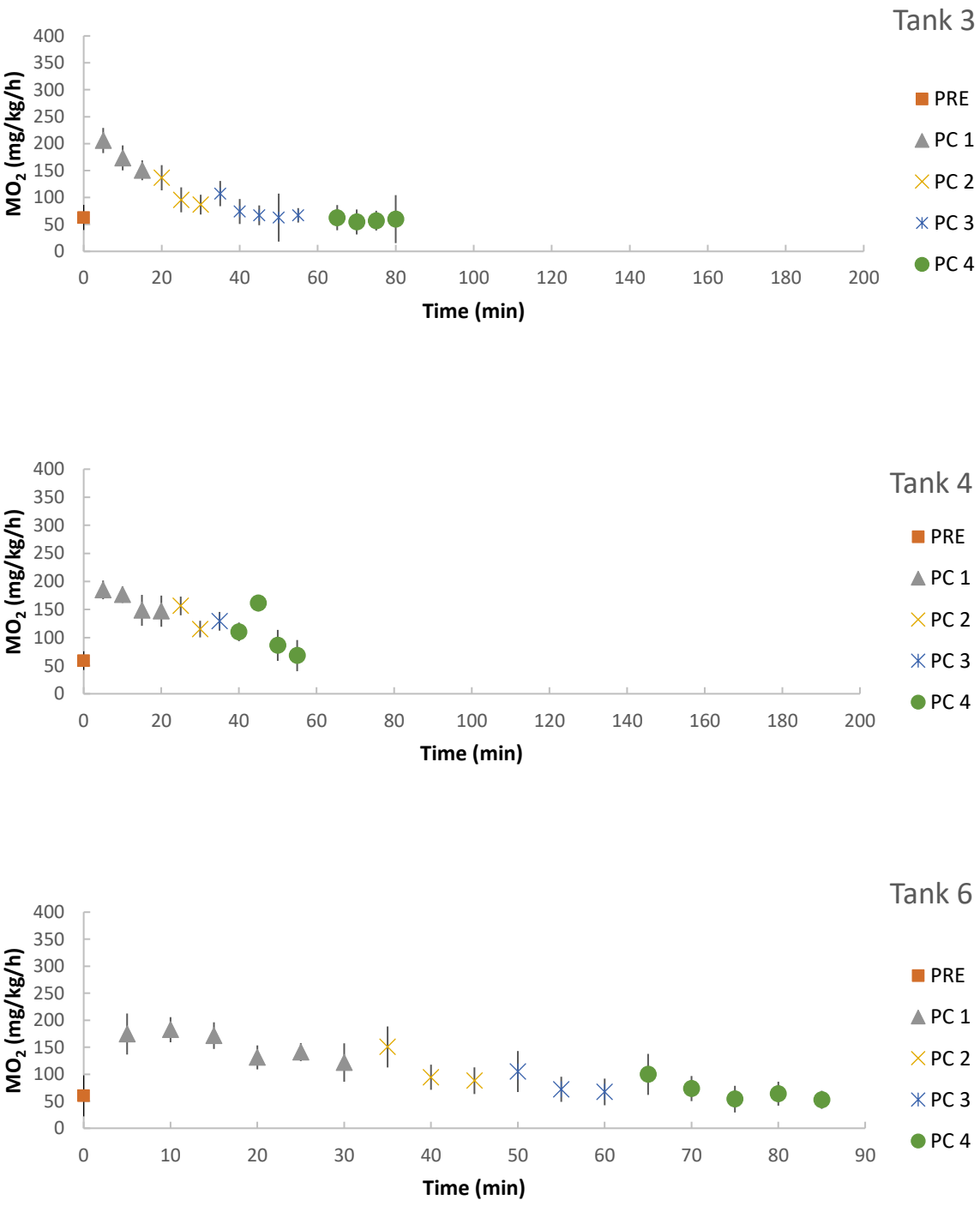

Tank 6
MO2 (mg/kg/h)
400
350
300
250
200
150
100
50
0
0
20
40
60
80
100
120
140
160
180
Time (min)
PRE
PC 1
PC 2
PC 3
PC 4

**Appendix Figure 2:** Atlantic salmon (*Salmo salar*) $MO_2$ values for individual ISAV+ tanks at 16 days post-infection, ISAV+ fish. Each point is the mean ± standard deviations of 5 values collected at 1 min intervals. Colours identify the different cycles measured in the respirometry analysis (PRE = pre-stress, PC1-4 = sequential post-stress cycles). EXPOC is represented by the area under this curve defined by the first point immediately after the PRE value to the first MO2 to come within 5% of the PRE value.



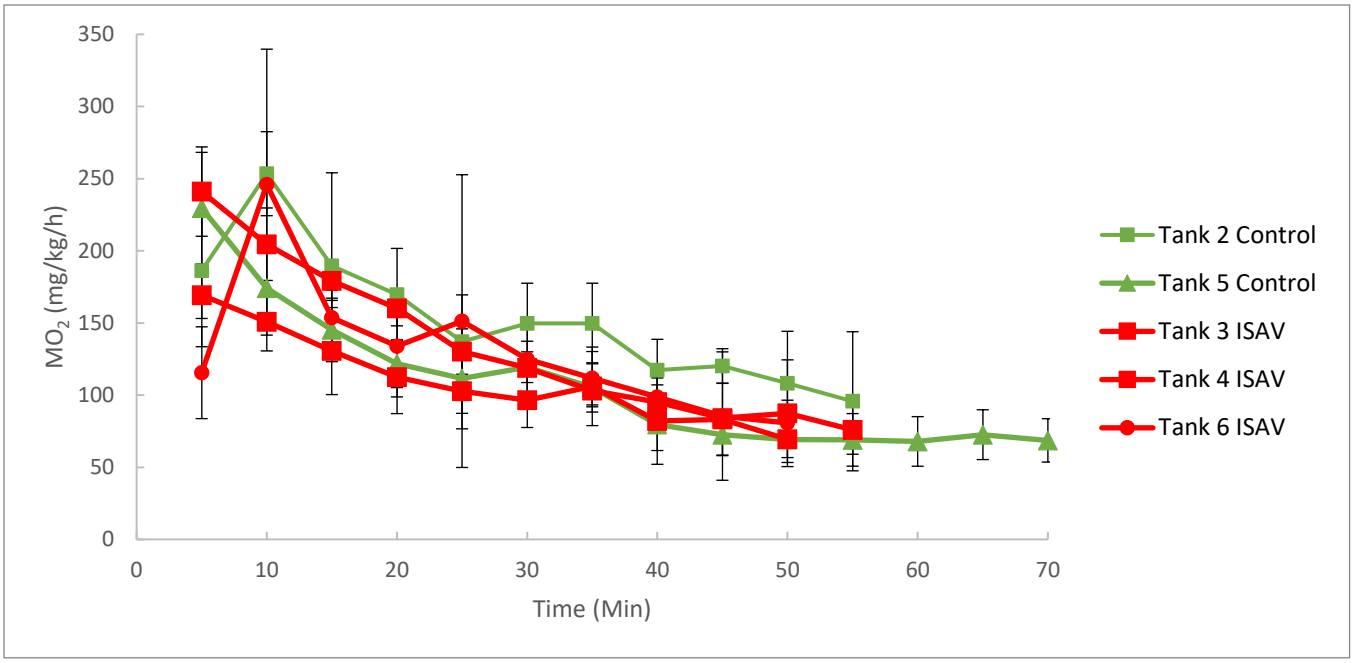

**Appendix Figure 3:** Atlantic salmon (*Salmo salar*) $MO_2$ values for individual control tanks at 30 DPI. Each point is the mean ± standard deviations of 5 values collected at 1 min intervals. Colors identify the

different cycles measured in the respirometry analysis (PRE = pre-stress, PC1-4 = sequential post-stress cycles). EXPOC is represented by the area under this curve defined by the first point immediately after the PRE value to the first $MO_2$ to come within 5% range of the PRE value.

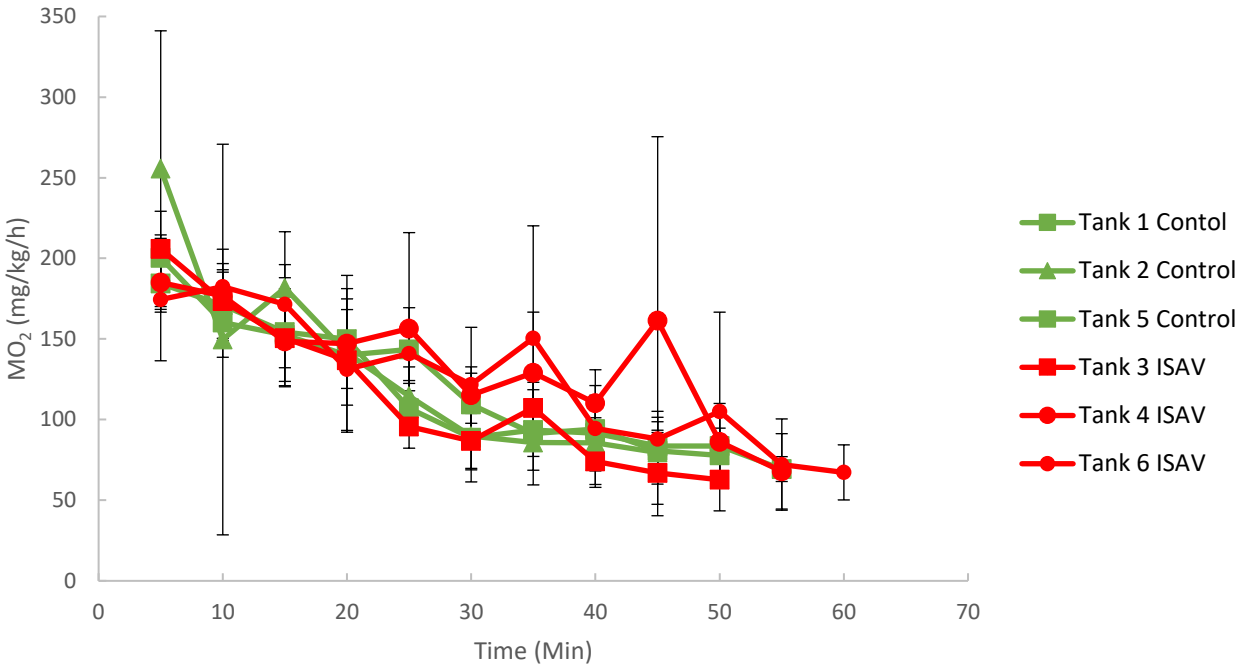



**Appendix Figure 4:** Atlantic salmon (*Salmo salar*) $MO_2$ values for individual ISAV+ tanks at 30 DPI. Each point is the mean ± standard deviations of 5 values collected at 1 min intervals. Colors identify the

different cycles measured in the respirometry analysis (PRE = pre-stress, PC1-4 = sequential post-stress cycles). EXPOC is represented by the area under this curve defined by the first point immediately after the PRE value to the first $MO_2$ to come within 5% range of the PRE value.




**Appendix Figure 5:** Segments of the post-stress cycles from all Atlantic salmon (*Salmo salar*) tanks (n = 2 control, n = 3 ISAV+) following a group respirometry analysis at 16 days post-infection. Sham-injected control (0.1 mL sterile HBSS) and ISAV+ (0.1mL of $10^5$ TCID 50/mL) of viral stock. These curves were used to calculate EXPOC by integrating the area under the curve. Each point is the mean ± standard deviations of 5 values collected at 1 min intervals. Tank 1 was excluded because of a malfunction.




**Appendix Figure 6:** Segments of the post-stress cycles from all Atlantic salmon (*Salmo salar*) tanks (n = 3 control, n = 3 ISAV+) following a group respirometry analysis at 30 days post-infection. Sham-injected control (0.1 mL sterile HBSS) and ISAV+ (0.1mL of $10^5$ TCID 50/mL) of viral stock. These curves were used to calculate EXPOC by integrating the area under the curve. Each point is the mean ± standard deviations of 5 values collected at 1 min intervals.

Appendix Table 1: Details of the Routine Metabolic Rate (RMR), Maximal Metabolic Rate (MMR), 105% of the RMR and the EXPOC values used to create Figure 9.

| Date | Tank | Status | RMR (mg/kg/min) | MMR (mg/kg/min) | 105% of RMR (mg/kg/min) | EXPOC (mg/kg) |
|---|---|---|---|---|---|---|
| 16 DPI | 2 | Control | 1.48 | 4.22 | 1.55 | 39.50 |
| | 5 | Control | 1.13 | 3.83 | 1.19 | 36.32 |
| | 3 | ISAV | 1.18 | 3.83 | 1.24 | 35.58 |
| | 4 | ISAV | 0.97 | 2.82 | 1.02 | 38.70 |
| | 6 | ISAV | 1.38 | 5.09 | 1.45 | 27.49 |
| 30 DPI | 1 | Control | 0.92 | 3.34 | 0.97 | 53.08 |
| | 2 | Control | 1.32 | 3.77 | 1.39 | 28.66 |
| | 5 | Control | 1.13 | 3.07 | 1.19 | 37.81 |
| | 3 | ISAV | 1.05 | 3.43 | 1.10 | 35.98 |
| | 4 | ISAV | 0.98 | 3.08 | 1.03 | 69.48 |
| | 6 | ISAV | 1.00 | 3.04 | 1.05 | 47.51 |

**Appendix Table 2:** Detailed breakdown of the number and stocking density of Atlantic salmon (*Salmo salar*) used for each of the separate experiments (Minimal Infectious Dose, Respirometry and Protein Synthesis Analysis). Table 2 in Section 2-3-2 has a detailed breakdown of the number of fish and the stocking densities for the respirometry experiment.

| Initial Number of fish | Experiment | Total number of fish | Mean body mass (g) | Tank Volume ($m^3$) | Mean Stocking Density($kg/m^3$) | Comments |
|---|---|---|---|---|---|---|
| 880 | Initial Receipt | 880 | 69.15 ± 29 | 1 | 11.00 ± 0.003 | Fish divided evenly in clean room holding tanks |
| | MID | 230 | 174 ± 16.2 | 0.275 | 12.65 ± 0.06<br>6.33 ± 0.06<br>12.65 ±0.06 | Positive Control n= 20<br>Negative Control n = 10<br>Virus bath n= 20 |
| | Respirometry | 252 | 261.3 ± 5.2 | 0.275 | | |
| | Protein Synthesis | 90 | 350± 48 | 1 | 0.35 ± 0.048 | All tanks n = 30 fish |
| | Holding tanks in clean room between experiments | Fish were held in changing numbers to maintain a density of 25.8 $kg/m^3$ | Fish were held in changing numbers to maintain a density of 25.8 $kg/m^3$ | | | |

**Appendix Table 3:** Detailed data for figures 4 and 13 of relative viral load observed in Atlantic salmon (*Salmo salar*) in two separate experiments (Respirometry and Protein Synthesis). Maximum, minimum and mean values.

| Variable | Experiment | Units | DPI | Max Value | Min Value | Mean |
|---|---|---|---|---|---|---|
| Relative Viral Load | Respirometry | RNA Copies | 16 | $10^{6}$ | $10^{3}$ | $10^{5.29}$ |
| | Respirometry | RNA Copies | 30 | $10^{5}$ | $10^{3}$ | $10^{4.70}$ |
| | Protein | RNA Copies | 7 | $10^{5}$ | $10^{3}$ | $10^{4.29}$ |
| | Protein | RNA Copies | 17 | $10^{6}$ | $10^{3}$ | $10^{5.49}$ |
| | Protein | RNA Copies | 78 | $10^{4}$ | $10^{2}$ | $10^{3.31}$ |

## Curriculum Vitae

Candidate's full name: Travis Ryan Melanson

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Baccalauréat ès Sciences Spécialisation en Biologie

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