Immunological and physiological effects of *Piscine orthoreovirus* infection in Atlantic salmon (*Salmo salar*)

Philosophiae Doctor (PhD) Thesis
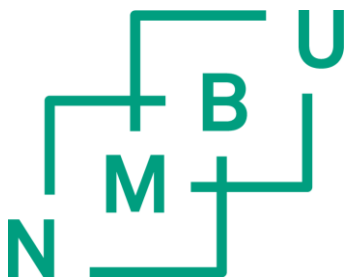
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Acknowledgements

Finally, I would express my gratitude to my family and friends for your support and patience during this work. A special thanks to my father and mother for your support, to Fabio for your happy mood and for dragging me outdoors and to my dear Julie for your endless sunshine and for keeping up with me at the finish line.

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Morten Lund

Abbreviations

APC   Antigen presenting cell

ATP   Adenosine triphosphate

CD4   Cluster of differentiation 4

CD8   Cluster of differentiation 8

CMS   Cardiomyopathy syndrome

CPE   Cytopathic effect

Ct   Cycle threshold

CTL   Cytotoxic T cell

DO   Dissolved oxygen

dsRNA   Double stranded RNA

EPO   Erythropoietin

f_{Hmax} Maximum heart rate

Hb   Hemoglobin

HCT   Hypoxia challenge test

HIF-1α   Hypoxia-inducible factor 1α

HSMI   Heart and skeletal muscle inflammation

HTC   Hematocrit

i.p.   Intraperitoneal

IFN   Interferon

Ig   Immunoglobulin

IHC   Immunohistochemistry

ISG   Interferon stimulated gene

mAbs   Monoclonal antibodies

MHC   Major histocompatibility complex

ODC   Hemoglobin-oxygen dissociation curve

pAbs	Polyclonal antibodies
PAMP	Pathogen associated molecular pattern
PD	Pancreas disease
PMCV	Piscine myocarditis virus
PRR	Pattern recognition receptor
PRV	*Piscine orthoreovirus*
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAV	Salmonid alphavirus
SPDV	Salmon pancreas disease virus
Topt	Optimum temperature
WBC	White blood cells

List of papers

Hypoxia tolerance and responses to hypoxic stress during heart and skeletal muscle inflammation in Atlantic salmon (*Salmo salar*)

Authors: Morten Lund, Maria Krudtaa Dahle, Gerrit Timmerhaus, Marta Alarcon, Mark Powell, Vidar Aspehaug, Espen Rimstad, Sven Martin Jørgensen

Revised version published: PLoS ONE 2017

Experimental *Piscine orthoreovirus* infection mediates protection against pancreas disease in Atlantic salmon (*Salmo salar*)

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Immunological interactions between *Piscine orthoreovirus* and *Salmonid alphavirus* infections in Atlantic salmon

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Summary

Heart and skeletal muscle inflammation (HSMI) is an important viral disease in Norwegian farmed Atlantic salmon (*Salmo salar*), and was associated with *Piscine orthoreovirus* (PRV) in 2010. Recently, the causality between PRV and HSMI was confirmed in Atlantic salmon experimentally infected with purified virus. Two important features of PRV infection are that erythrocytes are the most common target cell and the development of severe cardiac inflammation. Furthermore, the main function of erythrocytes and the heart is to transport oxygen (O_2) and carbon dioxide (CO_2) to and from metabolically active tissues. PRV is ubiquitously present in seawater-reared farmed Atlantic salmon in Norway, and the majority of HSMI outbreaks occur in the seawater grow-out phase. However, PRV infection also occur in freshwater reared pre-smolts, and HSMI outbreaks in pre-smolt facilities have been reported. The severity of HSMI outbreaks varies, and as for other infectious diseases, the outcome of PRV infection is influenced by agent-, host- and environmental factors. For example, handling of the fish and episodes of hypoxic seawater caused by environmental factors are common during the grow-out phase of the production cycle of Atlantic salmon. Little is known about the effects of PRV infection on hypoxia tolerance, or the side effects of additional stressors on HSMI development. The seawater-reared farmed Atlantic salmon are constantly exposed to numerous pathogens and co-infection of viruses is common. PRV-infected Atlantic salmon induces a long-lasting systemic antiviral immune response, which may affect the outcome of a secondary virus infection. Pancreas disease is caused by salmonid alphavirus (SAV) and is prevalent in Norwegian aquaculture in the southern half of the coastline. PRV and SAV have been co-detected in seawater farmed salmon and share target organs like the heart and skeletal muscle. This thesis aimed to elucidate some of these interactions by performing two challenge trials on PRV-infected Atlantic salmon.

The hypoxia tolerance of experimentally PRV infected Atlantic salmon was tested by progressively decreasing the oxygen saturation in the tank water. The PRV-infected fish showed significantly reduced hypoxia tolerance at two phases of the infection, one at peak virus levels in blood and heart, i.e. at 7 weeks post challenge, and one at peak inflammatory changes in the heart, i.e. at 10 weeks post challenge. This result was in line with the reduced cardiac performance observed in the PRV infected fish when measuring the maximum heart rate at time of peak inflammatory changes in the heart, which occurred at 10 weeks post challenge. At 7 and 10 WPI, the Hb levels were also reduced in the infected groups. At week 4, 7 and 10 after the onset of PRV exposure, one infected group was exposed to a 4 hour hypoxic episode to

study the effects of hypoxia on PRV infection. The pre-treatment by two transient hypoxic episodes improved the hypoxia tolerance tested at 10 weeks post challenge. Despite this, a reduced oxygen affinity was detected 10 weeks post challenge in erythrocytes from the PRV infected fish exposed to transient hypoxia. Taken together, these results show that PRV infection in Atlantic salmon lowers the tolerance to hypoxia, but previous exposure of transient hypoxia can counteract this effect without notable effects on HSMI development.

In another experimental trial, PRV-infected Atlantic salmon post-smolts were subsequently challenged by SAV 4 and 10 weeks post PRV challenge. The PRV infection reduced the outcome of the subsequent infection of SAV as observed by reduced viral load and severity of pancreas disease specific lesions. The protective effect of the PRV infection lasted for at least 10 weeks post PRV challenge. The protective effect was also observed in organs not known to be targeted by PRV, such as the pancreas. This indicated a systemic effect of the PRV-induced antiviral response. Gene expression analysis of blood, heart and spleen detected a significant up-regulation of antiviral immune genes following PRV infection. A plasma neutralization test detected SAV neutralizing activity in plasma from four PRV infected individuals sampled 4 weeks post PRV challenge. After inactivation of complement by heat treatment however, one plasma sample from a PRV infected individual still neutralized SAV.

Analysis of field data collected from PRV-SAV co-infected farmed Atlantic salmon detected a weak negative correlation between PRV and SAV RNA levels in clinically healthy individuals, in line with the conclusions of the experimental challenge. On the other hand, a weak positive correlation between SAV and PRV RNA levels was found in dead and moribund fish, showing that health status, i.e. immune status, is of importance for the outcome of a PRV-SAV co-infection.

In conclusion, the data presented in this thesis show that PRV infection of farmed Atlantic salmon have effects on the physiological and immunological responses of the fish. The PRV infection lowers the tolerance to hypoxia in Atlantic salmon, while the PRV infection mitigate the effect of subsequent infection with SAV, most likely through inducing long-lasting antiviral immune responses.

Sammendrag (Summary in Norwegian)

Hjerte og skjelettmuskel betennelse (HSMB) er en viktig virussykdom i Norsk oppdrett av Atlantisk laks (*Salmo salar*) og ble assosiert med *Piscine orthoreovirus* (PRV) i 2010. Wessel og medarbeidere bekreftet nylig årsakssammenhengen mellom PRV og HSMB i Atlantisk laks eksperimentelt infisert med renset virus. To viktige aspekter ved PRV infeksjon er at de røde blodcellene er den vanligste målcellen og utviklingen av alvorlig hjertebetennelse. Hovedfunksjonen til de røde blodcellene er å transportere oksygen (O_2) og karbondioksid (CO_2) til og fra metabolsk aktive vev. PRV er ubikvitært tilstede i sjøvannsoppdrettet Atlantisk laks i Norge og majoriteten av HSMB utbruddene skjer hovedsakelig i vekstfasen i sjø. PRV har blitt påvist i pre-smolt i ferskvannsanlegg i tillegg til rapporter om HSMB utbrudd i pre-smolt anlegg. Alvorlighetsgraden til HSMB utbruddene varierer og som for andre infeksjonssykdommer, er utfallet av PRV-infeksjon påvirket av agens-, vert- og miljøfaktorer. Håndtering av fisken og episoder med lave oksygenverdier i sjøen som følge av miljøfaktorer er eksempler på faktorer som er vanlig i løpet av vekstfasen til oppdrettet Atlantisk laks. Det er lite kunnskap om hvilke effekt PRV-infeksjon har på hypoksitoleranse eller hvordan utviklingen av HSMB påvirkes av andre stressfaktorer. Oppdrettet Atlantisk laks blir kontinuerlig eksponert for utallige patogener i sjøfasen, og påvisning av to ulike virus i samme fisk er vanlig. PRV-infisert Atlantisk laks induserer en langvarig systemisk antiviral immunrespons som kan påvirke utfallet av en sekundær virus infeksjon. Pankreassykdom (PD) er forårsaket av salmonid alphavirus (SAV) og er vanlig i Norsk akvakultur langs den sørlig halvdelen av kystlinjen. Ko-smitte av PRV og SAV har blitt påvist i sjøvannsoppdrettet laks, og begge virusene har hjerte- og skjelettmuskulatur som målorgan. Denne avhandlingen satte som mål å belyse noen av disse interaksjonene ved å gjennomføre to smitteforsøk på PRV-infisert Atlantisk laks.

Hypoksitoleransen til eksperimentelt PRV-infisert Atlantisk laks ble testet ved å gradvis redusere oksygenmetningen i vannet fisken ble holdt i. Den PRV-infiserte fisken hadde signifikant redusert hypoksitoleranse i to faser av infeksjonen; ved det høyeste nivået av virus i blod og hjerte (7 uker etter smitte) og ved den mest alvorlige graden av hjertebetennelse (10 uker etter smitte). Disse resultatene samsvarer med den reduserte hjertefunksjonen som ble observert i den smittede gruppen når maksimal hjerterate ble målt i fasen med mest alvorlig grad av hjertebetennelse, 10 uker etter smitte. Hemoglobinnivåene var også redusert i de infiserte gruppene 7 og 10 uker etter smitte. Ved uke 4, 7, og 10 etter PRV smitte ble en av de infiserte gruppene eksponert for 4 timer med hypoksi for å studere effektene hypoksi har på

PRV infeksjon. Eksponering for to perioder med hypoksi forbedret hypoksitoleransen når fisken ble testet 10 uker etter smitte. Til tross for dette, var oksygenbindingsevnen i blodet til den PRV infiserte fisken som også ble utsatt for perioder med hypoksi redusert ved uke 10 etter smitte. Kort oppsummert viser disse resultatene at PRV-infeksjon i Atlantisk laks reduserer toleransen for hypoksi, men at denne effekten kan motvirkes av tidligere eksponeringer for forbigående hypoksi uten å ha noen effekt på utviklingen av HSMB.

I det andre forsøket ble PRV-infisert post-smolt av Atlantisk laks eksperimentelt ko-infisert med SAV, 4 og 10 uker etter PRV smitte. PRV-infeksjonen reduserte utfallet av den påfølgende SAV infeksjonen ved å gi lavere virusnivå og alvorlighetsgrad av PD-spesifikke forandringer. Den beskyttende effekten av PRV-infeksjonen varte i opptil 10 uker etter PRV-smitte. I den ko-infiserte fisken kunne den beskyttende effekten observeres i organer som ikke er målorganer for PRV, som for eksempel bukspyttkjertelen (pankreas). Dette kan tyde på en systemisk effekt indusert av den antivirale responsen mot PRV. Analyser av genuttrykket i blod, hjerte og milt påviste en signifikant oppregulering av antivirale immungener som følge av PRV-infeksjonen En plasma nøytralisasjonstest viste nøytraliserende aktivitet mot SAV i plasma fra fire PRV-infiserte individer som ble tatt prøver av 4 uker etter PRV-smitte. Etter varmeinaktivering av komplement i plasma, viste fortsatt et individ nøytralisering av SAV.

I en analyse av feltdata bestående av PRV-SAV ko-infisert oppdrettet Atlantisk laks, ble det påvist en svakt negativ korrelasjon mellom PRV og SAV RNA nivåer i klinisk friske individer. Dette samsvarer med konklusjonene i det eksperimentelle studiet. Det motsatte ble imidlertid observert i ko-infisert syk fisk, hvor en svakt positiv korrelasjon mellom SAV og PRV RNA nivåer ble påvist og viser at helsestatus, dvs immunstatus, er viktig for utfallet av en PRV-SAV ko-infeksjon.

For å konkludere har dataene som er presentert i denne avhandlingen vist at en infeksjon med PRV påvirker de fysiologiske og immunologiske responsene i oppdrettet Atlantisk laks. PRV-infeksjonen reduserer hypoksitoleransen til Atlantis laks i tillegg til å redusere utfallet av en påfølgende SAV infeksjon, mest sannsynlig ved å indusere en langvarig antiviral immunrespons.

1   Introduction

1.1   General background

The Atlantic salmon (*Salmo salar*) aquaculture is an important industry. The production of Atlantic salmon constituted ~4.6 % in biomass and 14 % in value (in US$) of the global finfish aquaculture in 2014 (1). Atlantic salmon is produced in Norway, Chile, Scotland, Canada, USA, Faroe Islands, Ireland and Australia (Tasmania), where the first four countries are the major producers. Norway is by far the main producer in the world (~54 % of biomass) and exported 1.26 million tonnes farmed Atlantic salmon in 2014 (2).

Environmental factors and management factors such as high density of animals and artificial life cycles are contributors to the risk for outbreaks of infectious diseases in aquaculture (3,4). In the Norwegian Atlantic salmon aquaculture, infectious diseases and suboptimal environmental factors are causing mortalities and reduced animal welfare (5,6). Virus diseases poses a serious challenge in the Norwegian salmon aquaculture (7–10). However, the virus-induced disease outbreaks vary in mortality, indicating differences in virulence between virus strains or other elements that influence the disease outcome. Episodes of low oxygen content (hypoxia) in the environment during the production cycle (11) is an example of an environmental stressor that can affect the physiology (12) and immune response in Atlantic salmon (13).

Heart and skeletal muscle inflammation (HSMI) is an important viral disease in Norwegian aquaculture (6). *Piscine orthoreovirus* (PRV) is the causative agent of HSMI (Wessel et al, 2017, submitted PLoS One) and is detected in a high prevalence in seawater-reared farmed Atlantic salmon in Norway (14). Furthermore, PRV infects and replicates in the erythrocytes of the Atlantic salmon (15,16) and may influence the physiological and immunological functions of these cells. The primary task of the erythrocytes is to transport oxygen and carbon dioxide, and this function may be affected by the viral infection (17). They are also capable of mounting a strong antiviral immune response when infected (16–19).

There are several factors that may influence the outcome of a PRV infection in Atlantic salmon. Such factors may be stress, the state of the immune response, the infection pressure, virulence of the virus strain, co-infections with other pathogens, and water conditions like oxygen

saturation and temperature. Thus, studying the interplay between these factors may shed light on their effect on the outcome of a virus infection.

1.2   The life cycle of farmed Atlantic salmon

Most wild Atlantic salmon strains are anadromous and spend the first 1 - 6 years in the hatching river, and when they reach a specific body size, smoltification is initiated in early spring. The smoltification is a complex physiological process that prepares the freshwater adapted salmon parr to a saltwater environment during their migration to the sea. After a growth phase of 1 - 4 years in the northern Atlantic Ocean, the adult Atlantic salmon returns to the river of origin to spawn (Figure 1 A). In contrast, farmed Atlantic salmon is kept on land in fresh water tanks the first 8 – 16 months after hatching, before smoltification starts naturally or is induced artificially. The smolts are then transferred to open net pens at sea for the final grow-out phase that lasts 14 – 20 months until harvest (Figure 1 B).

A

The adult salmon return to their native stream in the spring for spawning

Adult male

Autumn: fertilized eggs are buried on the river bed

The growth phase in the Northern Atlantic Ocean lasts for 1 – 4 years

Adult female

Late spring: the eggs hatch and alevin nourish it self on the yolk sac

The parr smoltify in the spring and at around 12 cm body length. When smoltified, the smolts migrate to the ocean

3 – 6 weeks after hatching: the alevins starts to seek food and are called fry

The fry develop into parr and grow in their native stream for 1 – 6 years

B

Figure 1. *A. The lifecycle of wild Atlantic salmon (Salmo salar) from hatching to spawning. Modified from https://commons.wikimedia.org/w/index.php?curid=17848167. B. The production cycle of farmed Atlantic salmon from stripping to slaughter. Source: www.fao.org.*

The Norwegian Atlantic salmon marine aquaculture is located along the Norwegian coastline facing the North- and Norwegian seas (Figure 2).
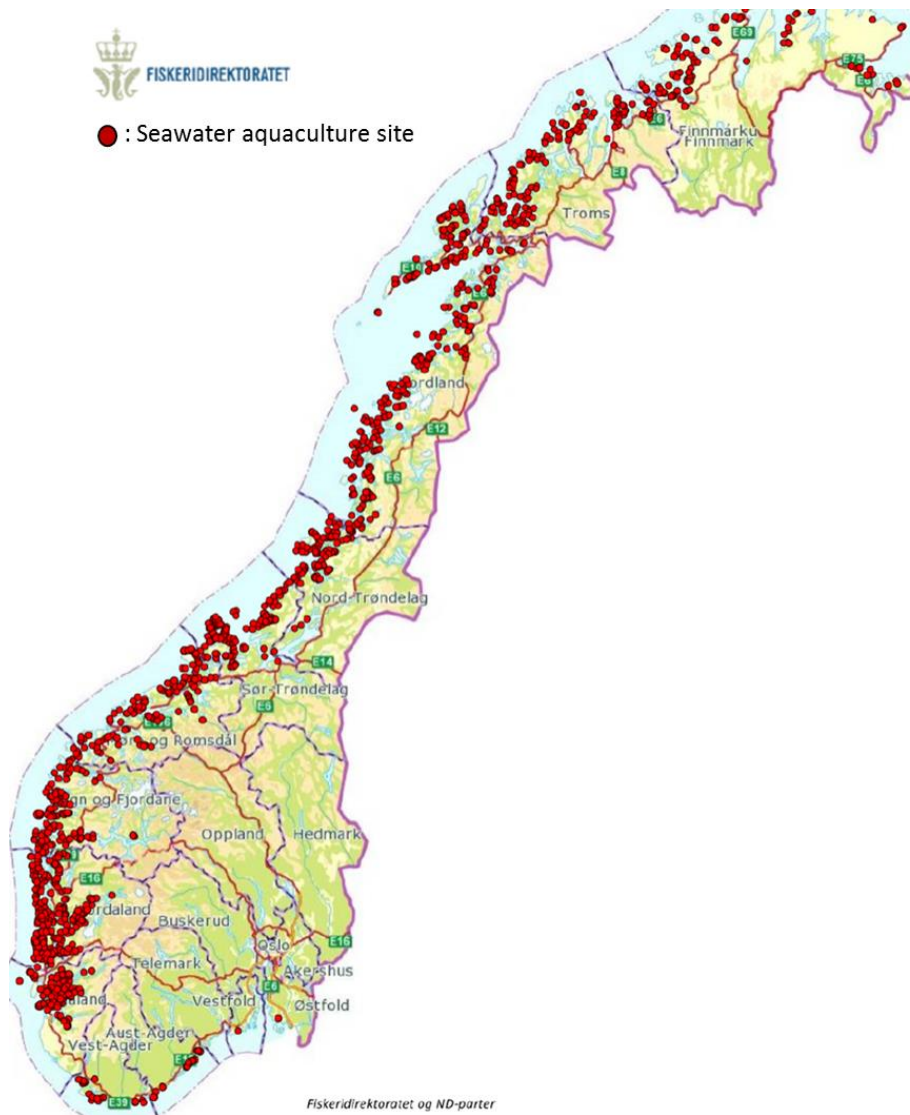
Figure 2. *Geographical location of seawater production sites (red dots) of Atlantic salmon. Source: The Norwegian Directorate of Fisheries, Bergen.*

1.3 Common infections in Norwegian aquaculture

Farmed Atlantic salmon are exposed to a wide variety of pathogens and emerging viral diseases poses a serious challenge in the Norwegian aquaculture (20). The most important infectious diseases in the marine Norwegian aquaculture from 2010 until today are listed in Table 1.

	2010	2011	2012	2013	2014	2015	2016
Infectious salmon anemia (ISA)	7	1	2	10	10	15	12
Pancreas disease (PD)	88	89	137	99	142	137	138
HSMI	131	162	142	134	181	135	101
Infectious pancreas necrosis (IPN)	198	154	119	56	48	30	27
Cardiomyopathy syndrome (CMS)	53	74	89	100	107	105	90
Salmon lice*	Total of 3115 treatments in 2016**						
Bacterial infections**	No frequency of outbreaks is available						
Amoebic gill disease (AGD)	No frequency of outbreaks is available						

Table 1. *Overview of the most important fish diseases in marine Norwegian aquaculture from 2010 to 2016. *: Lepeophtheirus salmonis, **: 1941 anti-parasitic drug treatments and 1174 non-drug treatments (ex. temperate water or fresh water), ***: ex. Moritella viscosa or Tenacibaculum spp. is most frequently detected. Source: The annual fish health report of the Norwegian Veterinary Institute (6).*

Co-infection is an infection of two or more un-related pathogens, (21) and a recent review showed that co-infections are common in teleost fish (22). The first viral co-infections in farmed salmonids were reported in rainbow trout in the USA (23) and Spain (24). Later reports have revealed that viral co-infections are common in farmed Atlantic salmon (25–29).

The ubiquitous presence of PRV in farmed Atlantic salmon makes co-infections with other viruses likely to occur. In Norway, co-infections have been reported between PRV and either salmonid alphavirus (SAV), Piscine myocarditis virus (PMCV) or Atlantic salmon calici virus (ASCV) (25,26,28–30). In Norwegian aquaculture, SAV and PRV cause disease and are present in the same geographical area, i.e. SAV is found south of Nordland county while PRV is present all along the Norwegian coast (6). The immunological interactions during a PRV-SAV co-infection in Atlantic salmon is thus important to understand better.

1.4 *Piscine orthoreovirus* and heart and skeletal muscle inflammation

1.4.1 *Piscine orthoreovirus* (PRV)

PRV is a non-enveloped virus containing a double stranded (ds) RNA genome of ten segments, and taxonomically placed in the *Reoviridae* family, genus *Orthoreovirus* (31,32). The prevalence of *Piscine orthoreovirus* is high (55 – 100 %) in farmed, seawater-reared Atlantic salmon and also common in wild salmon along the Norwegian coast where Atlantic salmon is farmed (14,30,33). In addition to Atlantic salmon, PRV or PRV-like viruses have been detected in rainbow trout (*Oncorhynchus mykiss*), Sea trout (*Salmo trutta*), Coho salmon (*Oncorhynchus kisutch*) and Sockeye salmon, in both farmed and wild populations in Canada, Chile (14,34–38). Futhermore, PRV has also been described in farmed Atlantic salmon in Ireland and in farmed Coho salmon in Japan (39,40)

PRV infection in erythrocytes

The piscine erythrocytes are nucleated and contain functional organelles (41) which allow aerobic metabolism, transcription and translation (18,42,43). The transcriptional machinery of the erythrocytes can also support virus propagation. Finstad and co-workers have shown that erythrocytes are important target cells for PRV (15). Figure 3 shows immunofluorescent staining of the PRV capsid protein σ1 in *in vivo* infected salmonid erythrocytes. During peak infection, more than 50 % of the erythrocytes can be infected, as measured by flow cytometry (15). PRV has also been shown to infect and replicate in erythrocytes *ex vivo* (16). Furthermore, the salmonid erythrocyte is able to mount an innate immune response (18,19), which is also initiated after a PRV infection (16,17). This may suggest that the nucleated erythrocytes play an important role in antiviral immunity in salmonids (44).
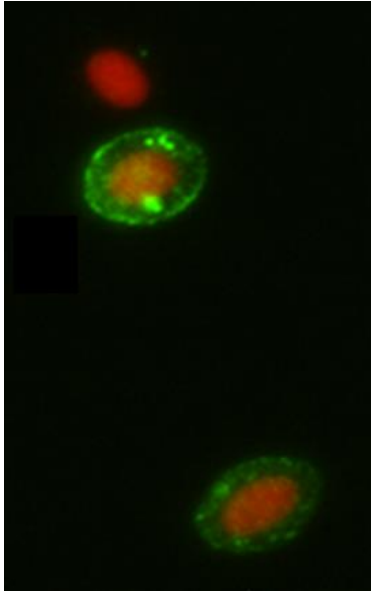
Figure 3. *Immunofluorescent staining of the PRV outer capsid protein σ1 (green) in infected Atlantic salmon erythrocytes. The erythrocyte nucleus is colored red. Photo: Øystein Wessel.*

Challenge studies have shown that the PRV infection persists in blood cells, heart, spleen, liver and brain with relatively high production levels of viral RNA (15,38,45–47), up to 59 weeks post challenge (48). Furthermore, PRV-infected erythrocytes showed a general suppression of non-immune genes (17). These results shows that the Atlantic salmon erythrocytes respond to PRV infection, at least by altered transcription and translation. The primary function of the erythrocytes is to transport oxygen and carbon dioxide, and the effect of a PRV infection on these functions was unknown at the beginning of this PhD project.

1.4.2 Heart and skeletal muscle inflammation

Heart and skeletal muscle inflammation (HSMI) was first detected in diseased farmed Atlantic salmon in Norway in 1999 (49). Since then, the number of outbreaks registered by the Norwegian Veterinary Institute (NVI) has been high (Figure 4) and proves HSMI to be one of the most frequently diagnosed viral diseases in the last decade in Norway (Table 1). Furthermore, HSMI is considered as an important and emerging viral disease in Norwegian aquaculture (20). In 2014, HSMI was removed from the list of fish diseases notifiable to the Norwegian Food Safety Authority and thus, since 2015 the number of outbreaks registered by the NVI are therefore minimal numbers.
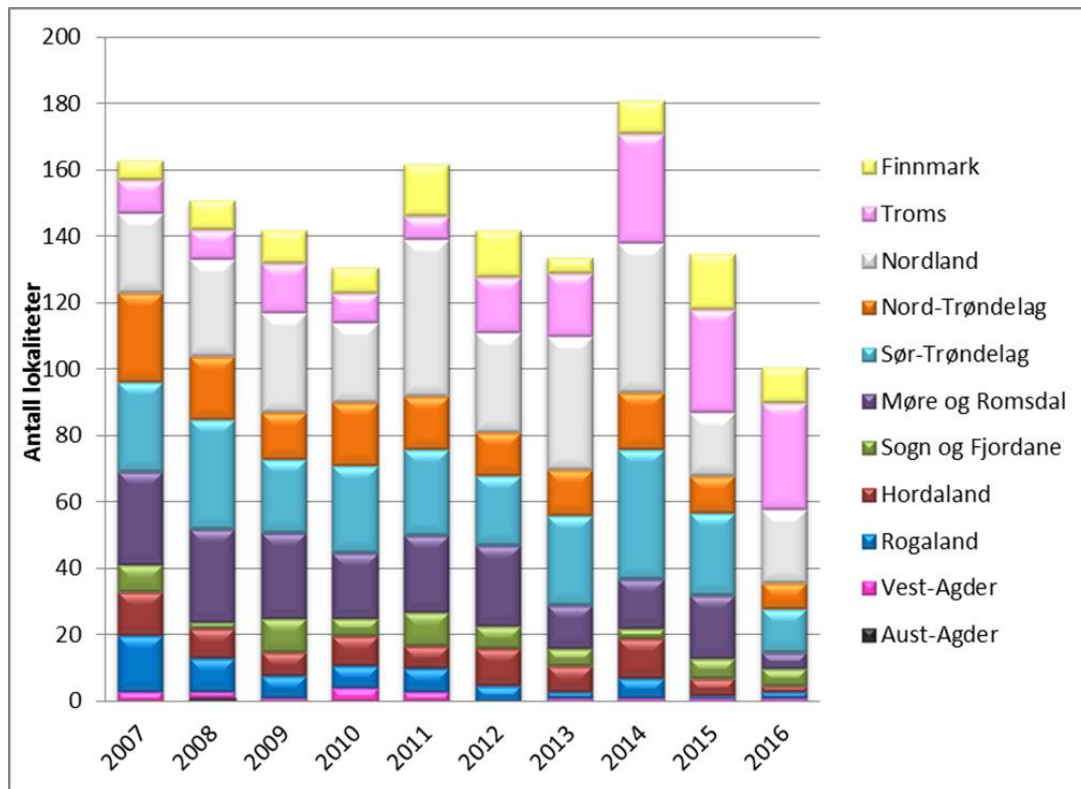
Figure 4. *The total and county wise number of annually HSMI outbreaks in Norway form 2006 - 2016. Source: The annual fish health report of the Norwegian Veterinary Institute* (6).

In 2010, HSMI was associated with *Piscine orthoreovirus* (PRV) (31) HSMI outbreaks are distributed along the Norwegian coastline harboring aquaculture facilities (6) (Figure 5). HSMI has also been reported in farmed Atlantic salmon in Scotland, Chile and BC Canada (50–52), which confirms the significance of a PRV infection in other Atlantic salmon farming countries.
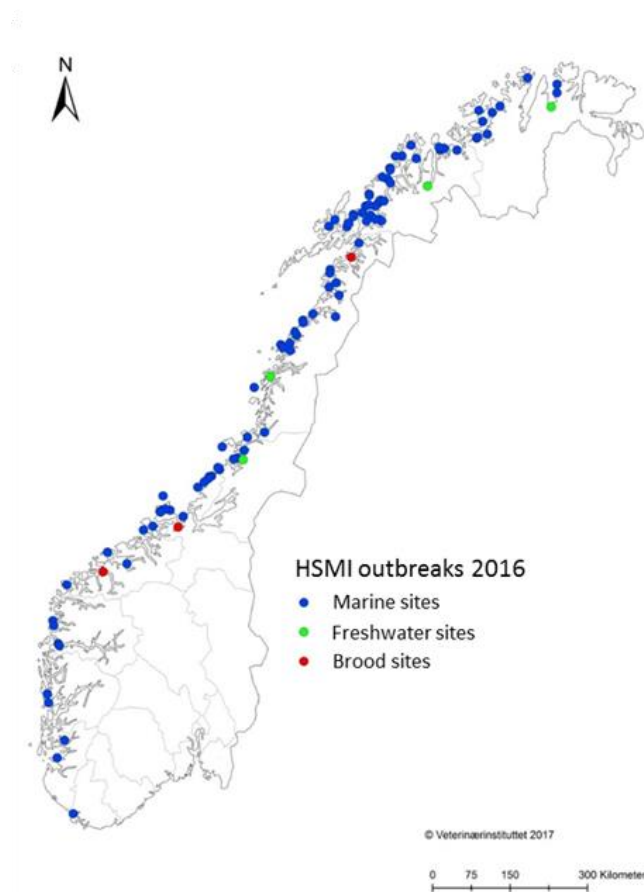
Figure 5. *The geographical distribution of HSMI outbreaks in Norway in 2016. Blue dots indicates seawater sites, green dots indicates freshwater, pre-smolt facilities and red dots indicates brood fish sites. Source: The annual fish health report of the Norwegian Veterinary Institute* (6).

HSMI outbreaks usually occurs 5 - 9 months after seawater transfer and are clinically characterized by reduced appetite, lethargic fish and a varying degree of mortality (53). The disease has been detected as early as 14 days after seawater transfer and recently, in pre-smolt freshwater facilities (6,14,53). The accumulated severity of HSMI outbreaks in the marine production phase varies from negligible to 20 % (54). Post-mortem investigations show well-fed individuals having an average to large body mass, but a pale heart, varying degree of ascites and/or pericardial fluid, discolored or yellow liver, swollen spleen and petechiae in the perivisceral fat (49,53) (Figure 6).

Figure 6. *Macroscopic pathologically findings of a HSMI diseased adult, farmed Atlantic salmon. Photo: Trygve Poppe (Pharmaq Analytiq AS)*

The HSMI diagnosis is based on clinical signs and histopathological changes in the heart and skeletal muscle. Important differential diagnoses to HSMI is pancreas disease (PD) and cardiomyopathy syndrome (CMS), however these diseases are differentiated histopathologically (55) (Table 2).

Organ	Lesions description	CMS	HSMI	PD
Heart	Epicarditis Compact-myocarditis and degeneration Spongy-myocarditis and degeneration	+ - +	+ + +	+ + +
Skeletal muscle	Inflammation and degeneration	-	+	+
Liver	Necrosis of hepatocytes	-	-	+
Pancreas	Necrosis of exocrine tissue	-	-	+

Table 2. *The table shows different histopathological finding of CMS (cardiomyopathy syndrome), HSMI (heart and skeletal muscle inflammation) and PD (pancreas disease). + indicates findings of the particular disease, - indicates this not being a finding. Modified from Yousaf et al, 2013 (55).*

HSMI pathogenesis

Due to the infection of the erythrocytes, PRV is transported to every blood perfused organ. The initial histopathological finding of HSMI is infiltration of mononuclear cells in the epicardium. The epicarditis is followed by a severe infiltration of mononuclear cells and myocardial necrosis

in the compactum and spongiosum (45,56,57) (Figure 7). The infiltration of the mononuclear cells is the basis for scoring of the severity of HSMI during challenge trials. Finally, the panmyocarditis reach peak levels approximately at 10 weeks post PRV challenge in experimental cohabitation models (15, Paper I, Paper II) and is gradually reduced within 4 weeks thereafter (Paper I). In severe cases, cell infiltration in red skeletal muscle, the atrium and liver is also detected (53,54). In field outbreaks, the inflammatory changes in the heart may last for several months within a population of farmed Atlantic salmon before fading off (52,54). By immunohistochemical staining the PRV antigens are initially present in blood cells and subsequently found in cardiomyocytes in the heart ventricle (15).
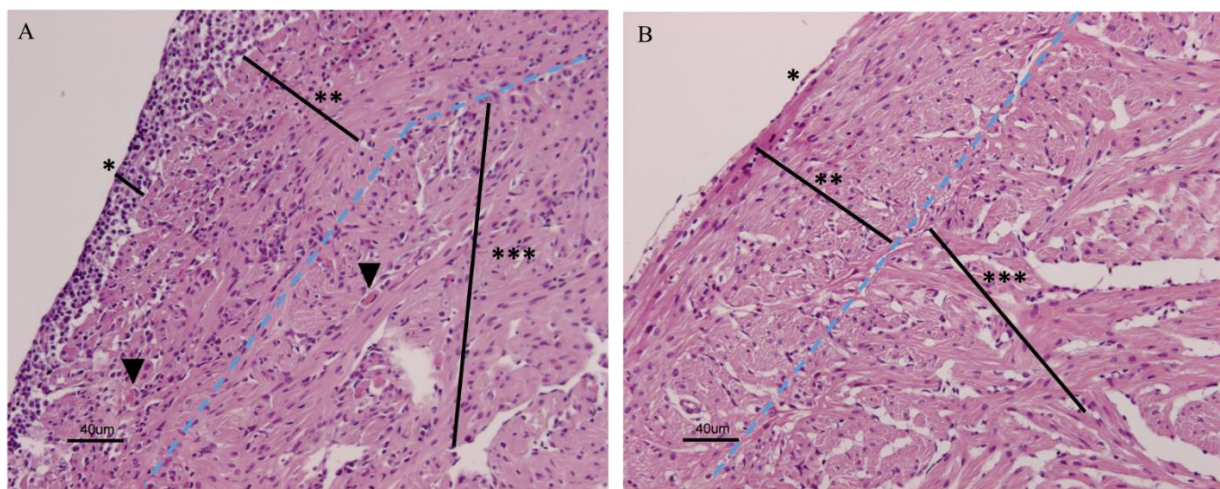
Figure 7. *A. Picture of epicarditis and myocarditis from a heart having an average histopathological cardiac inflammation score (1.86). The score ranged from 0 (no inflammatory changes) to 3 (severe panmyocarditis). The PRV RNA Ct values in heart and blood of the fish in A were17.2 and 20.5, respectively. B. Picture of a non-infected heart. The hearts in A and B are sampled at 7 weeks post PRV challenge (Paper I). * indicate epicardium, ** indicate compactum, *** indicate spongiosum, arrowhead indicate myocardial necrosis. Bars indicate the respective layers, which is seperated by a blue dashed line. (Magnification: 20x) Photos: Morten Lund*

1.5   Responses to hypoxia

PRV is ubiquitous in seawater-reared Atlantic salmon in Norway and infect over 50 % of the erythrocytes at peak phase of infection. Hence, there is a need to elucidate if this could influence the capacity for oxygen transport and the hypoxia tolerance of affected fish. Furthermore, the panmyocarditis and epicarditis characteristic for HSMI may compromise the cardiac performance. The aim of Paper I was to investigate the hypoxia tolerance, cardiac performance and hemoglobin-oxygen affinity of PRV-infected Atlantic salmon. The following sections provide an overview on the cardiorespiratory physiology and the responses to environmental hypoxia in teleost fish.

1.5.1   Introduction to cardiorespiratory physiology in teleost fish
An overview on the metabolic rate in teleost fish

Oxygen is essential for cellular respiration, i.e. the conversion of the energy from food into adenosine triphosphate (ATP). ATP is normally produced by oxidative phosphorylation in the presence of sufficient oxygen, and fuels the basal cellular functions like ion pumping across cell membranes, osmoregulation and protein synthesis. These functions constitutes the basal metabolism, i.e. the standard metabolic rate (SMR), of an organism and are necessary for sustaining cellular homeostasis and survival (58). Ambient temperature is a major factor controlling the metabolic rate in teleost fish (59,60), but also salinity, body mass and developmental stage affects the SMR in teleosts (61). An increase in SMR due to elevated water temperature have been detected in salmonids (62,63), which leads to a higher demand for ATP, and hence a higher oxygen consumption rate. When the ATP requirements for the basal cellular functions are covered, the excess energy can be utilized for growth, reproduction and locomotion and constitutes the routine metabolic rate (RMR) of the organism (64). Thus, increased digestion, swimming activity or physical stress, will all increase the RMR and oxygen consumption rate. The maximum metabolic rate (MMR) is the metabolic rate at the maximal oxygen uptake of an animal. The difference between SMR and MMR is termed the aerobic scope for activity of the fish (65). The aerobic scope may be reduced either by an increase in SMR (due to increased water temperature or osmoregulation), by an increase in RMR (ex. due to increased digestion, swimming or growth) or by a reduction in MMR (ex. due to environmental hypoxia) (66,67). Hence, the lower aerobic scope - the lower potential of increasing the aerobic metabolism to be used for physical activity. The temperature at which the aerobic scope is greatest is termed the optimum temperature (T_{opt}), and the heart rate (f_H) responds to increased temperature in teleost fish. At water temperatures above T_{opt} for aerobic

scope, the heart rate decreases and causes the aerobic scope to fall (68). The T_{opt} for aerobic scope has shown to vary between families and species of salmonids (68–70).

Oxygen transport in the teleost erythrocytes

The gills in teleost fish extract oxygen from the environment. Fish access O_2 from the surrounding water by creating a one-way flow of water over the gills through the movements of the buccal and opercular pumps. The exchange of O_2 and CO_2 between water and blood occurs at the gill lamella by diffusion across the gill epithelium and into the erythrocytes. The unidirectional flow of blood within the capillaries in the gill lamella is the opposite to the water flow across the gills. This countercurrent flow of blood and water at the gill lamella creates an oxygen concentration gradient, which optimizes the oxygen uptake from the water (71). The oxygenated blood from the gills is transported via arteries to the metabolically active tissues. The oxygen is transported in the blood reversibly bound to hemoglobin (Hb) in the erythrocytes. Hemoglobin is a tetramer of four globin subunits (two α- and two β-subunits), each of which contains one heme-group that is the binding site of the oxygen molecule (72). The α- and β-globin genes are conserved among vertebrates, which confirms the evolutionary importance of hemoglobin in oxygen transport (73).

The oxygen-carrying capacity of the blood is dependent on the Hb concentration in blood and the affinity of Hb for oxygen. The hemoglobin-oxygen (Hb-O_2) affinity describes how easily hemoglobin binds and releases oxygen and is normally quantified by determining the P_{50} value, i.e. the partial pressure of oxygen (PO_2) at which Hb is 50 % saturated with oxygen. The P_{50} value can be derived from an oxygen dissociation curve (ODC). The ODC is a graphical presentation of the oxygen saturation of Hb relative to the blood PO_2 and illustrates the oxygen binding properties of hemoglobin (72). A shift of the ODC to the left, which occurs during hypoxia in teleost fish, indicates an increased Hb-O_2 affinity and thus an increased loading of oxygen in the gills (74). The Hb-O_2 affinity is affected by the intra-erythrocytic pH (75). In situations which increases CO_2 levels and low pH in peripheral blood (ex. during strenuous exercise), the P_{50} value increases and is detected as a right shift of the ODC (76). A right shift of the ODC reflects a reduced Hb-O_2 affinity and results in an increased unloading of oxygen in the tissue (72). The effect on Hb-O_2 affinity due to changes in blood pH is known as the Bohr effect (77) and is detected as either a right or left shift of the ODC curve (78). The hemoglobin molecules of some fish species have a unique property called the Root effect. The Root effect is a reduced Hb-O_2 carrying capacity due to a sudden drop in blood pH and inhibits the fish

blood to reach close to 100 % oxygen saturation at atmospheric PO_2 (which normally ensures full O_2 saturation of Hb). The Root effect is important to fish because it allows filling of oxygen in the swim bladder and ensures oxygen supply to the retina, which lacks capillaries to facilitate O_2 delivery as in other tissues (79).

The intra-erythrocytic organic phosphates are also important factors affecting the Hb-O_2 affinity and are conserved among vertebrates (80). In teleost fish, a reduced intra-erythrocytic concentration of nucleoside triphosphates (primarily adenosine triphosphate (ATP) and guanosine triphosphate (GTP)) increases the oxygen affinity of the Hb molecules (75,81).

The teleost erythrocytes – an overview of erythropoiesis and cellular metabolism
The erythrocytes in teleost fish are ellipsoidal shape and contain an oval nucleus. Normally, a teleost erythrocyte have a half-live of around 50 days (82) and the hematocrit (volume percentage of erythrocytes in blood) may range from 23 – 49 % (83,84). The circulating erythrocyte population of teleost fish consists of immature/young and mature cells. In rainbow trout blood, 81.9 %, 10.6 % and 7.5 % of the erythrocytes were categorized as mature, immature and post-mature, respectively (85).

The development of erythrocytes, i.e. erythropoiesis, is conserved among vertebrates (86). In teleost fish, erythropoiesis occurs in the kidney and spleen (87,88). The spleen serves additionally as a storage for mature erythrocytes (89). As in mammals, erythropoiesis in teleost fish is initiated by the hormone erythropoietin (EPO) (90), which gene is mainly expressed in the teleost heart in response to anemia and hypoxia (90–92). In mammals and teleost fish, EPO acts by activating the JAK/STAT5 signaling pathway in the erythroid progenitor cells to halt apoptosis (controlled cell death) of the cells (see section 1.6.3 for more details on the JAK/STAT pathway). This allows the erythroid progenitor cells to continue and complete their development and become mature erythrocytes (86,90). The expression of the EPO gene is regulated by the hypoxia-inducible transcription factors (HIFs), which is upregulated in response to hypoxia. HIFs also initiates transcription of genes involved in globin synthesis, angiogenesis and altering of the gill surface (93).

In contrast to the enucleated mature mammalian erythrocytes, the erythrocytes of birds, reptiles, amphibians and fish contains a nucleus and functional organelles (41), which facilitates protein synthesis and aerobic metabolism. The mammalian erythrocytes are mainly dependent on

anaerobic, i.e. oxygen-independent, energy production (94), while the teleost erythrocytes produce their energy in an oxygen-dependent way (42). The cellular metabolism of the Atlantic salmon erythrocytes relies on aerobic respiration (95). Furthermore, rainbow trout erythrocytes produces 99 % of their energy aerobically in normoxic conditions (96). This indicates that the salmonid erythrocytes relies on a sufficient oxygen supply to function normally. Futhermore, the mature teleost erythrocytes are able to synthesize proteins and seems to continue to produce Hb throughout their lifespan (85,97). However, the gene transcription level and protein synthesis in the old rainbow trout erythrocytes were lower compared to young erythrocytes. Nevertheless, no differences in total protein levels were detected between young and old erythrocytes (98).

The salmonid heart – a brief overview of the cardiac anatomy
The pumping action of the heart secures a steady circulation of blood and thus oxygen delivery to the metabolically active tissues. The salmonid heart consists of three chambers; the atrium, ventricle and the bulbus arteriosus (99). The epicardium and endocardium consist of one layer of flattened epithelial cells and covers the outer and inner borders, respectively, of the heart chambers. The atrium consists of a thin layer of smooth muscle cells. The cardiac ventricle wall consist of two layers; the compactum and spongiosum. The compactum is the outer layer of the ventricle wall and consists of cardiomyocytes in a circumferential arrangement. The spongiosum is the inner most layer and made up of a muscular trabeculae (99). The bulbus arteriosus consists mainly of elastin, smooth muscle cells and collagen and is not contractile.

The oxygen supply to the compactum of the salmonid ventricle is delivered by arterial blood from the gills via the coronary arteries and the spongious layer receives its oxygen from the venous blood entering the ventricle lumen (100).

Environmental hypoxia
Gill breathing encounters different challenges regarding ventilation compared to air breathing. In comparison to air, the physical properties of water, i.e. higher density and viscosity, slower diffusion rate of oxygen and lower solubility of gasses, increases the energy cost of ventilation and decreases the availability of oxygen to gill-breathing fish (101). The amount of dissolved oxygen (DO) in the arterial blood leaving the gills closely resembles the level of DO in the water given an optimal Hb-O_2 affinity (102). Therefore, a reduced oxygen content in the surrounding waters will reduce the DO in the blood, and may thereby affect cellular respiration.

Environmental hypoxia is the level of DO in the water that causes hypoxemia, reduces aerobic scope and compromises the metabolism in the fish (103). A progressive increasing hypoxemia triggers several compensatory mechanisms in order to sustain the oxygen consumption rate and an aerobic ATP production (section contains 1.5.2 more details on the responses to hypoxia). However, at the level of dissolved oxygen when the cellular ATP production changes from aerobic to anaerobic, i.e. the critical oxygen level (O_{2crit}), the SMR is no longer sustained by aerobic respiration and the aerobic scope is zero (103). Thus, at oxygen levels below O_{2crit} the cellular functions and homeostasis is compromised.

The amount of dissolved oxygen in seawater (10°C, 35‰ salinity) is 8.6 mg/L when equilibrated to air (104). At Atlantic salmon seawater production sites, the level of dissolved oxygen fluctuates due to various reasons (105–108). The fluctuations in DO levels are mainly due to environmental factors such as variations in water temperature and salinity, water currents, a stratified water column, photosynthesis by algae and a respiring microflora, as well as the oxygen consumption of the fish in dense populations (104,105,109,110). The variations in oxygen level at Atlantic salmon sites can be acute or subtle and vary diurnally, weekly, monthly and seasonally (106,108,111). Additional factors such as biofouling and increased oxygen consumption during feeding, handling and crowding, have caused fluctuations in the oxygen saturation level between 30 - 100 % in seawater net pens containing farmed Atlantic salmon (11,105,106,108,111). Furthermore, an increase in the RMR and thus increased oxygen consumption rate of fish occurs during physical activity or stress (112). In Atlantic salmon farming, such physical stress factors may be crowding or handling of the fish related to de-lousing, an inherent high growth-rate and a high fish density or high biomass in the net pens, which may affect the oxygen content within the net pen.

Evolutionary adaptation to hypoxic habitats in teleost fish

Teleost fish species constitutes over half of the vertebrate species, and display a high biological and genetic diversity (113,114). The diversity has allowed teleost fish to adapt to and survive in habitats highly varying in dissolved oxygen. To cope with hypoxic environments, teleost fish have evolved various metabolic, physical and physiological strategies to sustain adequate oxygen uptake and supply to the metabolically active tissues (115). An example of evolutionary environmental adaptation, is the Antarctic icefish (family *Channichthyidae*) lacking both erythrocytes and hemoglobin. In these fish, the oxygen is transported in a dissolved state in the plasma (116). This way of supplying the tissues with oxygen is an adaptation to an aquatic

environment having stable temperatures below 0 °C and a high oxygen content (117). Air breathing and hypoxia-tolerant teleost fish species are other examples of evolutionary adaptations to hypoxic or anoxic habitats (117). The gill remodeling of the crucian carp (*Carassius carassius*) in response to hypoxia represents yet another evolutionary adaptation in order to handle varying environmental oxygen supply (118). In normoxic conditions, the space between the gill lamella of the crucian carp is filled with a cellular mass, increasing the diffusion distance of oxygen into the blood. However, when encountering hypoxic waters, the interlamellar cell mass is retracted and hence, reduces the diffusion distance of oxygen (118).

1.5.2 Responses triggered by hypoxia in teleost fish

During the seawater grow-out phase, the wild Atlantic salmon is a pelagic and active species. Furthermore, a high growth rate in Atlantic salmon suggest a high metabolic rate, which requires a sufficient supply of oxygen (119). Salmonids are regarded as hypoxia intolerant fish (120). Atlantic salmon that encounters acute environmental hypoxia may try to distance themselves from the hypoxic waters. However, farmed Atlantic salmon are confined in a seawater net pen and thus have limited mobility. During environmental hypoxia, several physiological mechanisms are initiated to compensate for the reduced oxygen availability and to sustain the metabolic needs for ATP. In the coming section, some of the responses to hypoxia in teleost fish are included.

Reduced oxygen consumption

During environmental hypoxia, the oxygen consumption rate is reduced by lowering the RMR, i.e. by reducing the feeding rate (121) and swimming activity (67). However, if the oxygen levels fall below the O_{2crit} of the fish, oxygen-independent ATP production is initiated (anaerobic respiration) (67). Anaerobic respiration yields only two molecules of ATP per glucose molecule. Compared to the ~30 ATP molecules per glucose molecule gained from aerobic respiration, the anaerobic respiration reduces the energy supply to the cells significantly. Furthermore, anaerobic ATP production uses glycogen as the main glucose source and the amount of glycogen available is therefore a limiting factor for how long anaerobic respiration can be sustained (122). An eventual halt in ATP production due to lack of substrate and oxygen will result the inability to maintain cellular homeostasis and eventually cell death will occur (123). Thus, the survival of the fish depends on its capacity to reduce the ATP consumption rate, i.e. lowering the metabolic needs, and on the glycogen storage of the

organism available for anaerobic respiration (123). In addition, the hypoxia-induced anaerobic glycolysis produces lactate (124,125). Accumulation of lactate has also been suggested to be the cause of death during severe long-term hypoxia or anoxia in teleost fish (126).

Improved blood oxygen uptake and transport

One of the initial responses to environmental hypoxia in fish is to increase gill ventilation rate and depth that increase the amount of water that flows over the gills per minute and to increase the perfusion of blood in the gills (127,128). Futhermore, the hyperventilation causes a respiratory alkalosis (increased blood pH), which increases the Hb-O_2 affinity (129,130). This will increase the oxygen uptake from the environment. However, the high energy costs of hyperventilation in water entails that this strategy is not cost-efficient during long-term and severe hypoxia (131). The oxygen-carrying capacity may also be increased by the release of erythrocytes from splenic contraction (68,132,133), or by increased erythropoiesis (86,134,135), which is initiated by hypoxia-inducible factor 1α (HIF-1α) (93). In teleost fish, upregulation of the genes encoding EPO and the EPO receptor (EPOR) have been detected in the heart and spleen in response to hypoxia and anemia (86,91,92). HIF-1α also initiates transcription of several genes involved in globin synthesis, angiogenesis and altering of the gill surface (93), all of which improves the hypoxia tolerance of the fish. Piscine erythrocytes respond to stress stimuli such as hypoxia by swelling, which reduced the concentration of Hb and ATP within the cells and increases the Hb-O_2 affinity (74,129).

A common consequence of hypoxia in teleost fish is a reduced intra-erythrocytic concentration of the organic triphosphates ATP and GTP. In rainbow trout exposed to hypoxia, the concentrations of ATP and GTP in the erythrocytes was reduced (74,136). A reduced intra-erythrocytic concentration of ATP increases the hemoglobin-oxygen affinity and hence increases the oxygen-carrying capacity of the erythrocytes (74,75,81). The increased Hb-O_2 affinity improves the oxygen loading of the blood at the gills. Furthermore, in the metabolic active tissues, high levels of CO_2, lactate and H^+ accumulates during hypoxia, strenuous exercise or stress (125,137). This lowers the pH in the peripheral blood and reduces the Hb-O_2 affinity, which increases the O_2 unloading in the tissues (137).

Stress response to hypoxia

A stress response in fish can be triggered by exposure to physical (ex. crowding or handling) or chemical (ex. salinity, hypoxia, and chemicals) stressors, or by a fear reaction. The stress

responses are characterized by the secretion of catecholamine hormones (adrenaline and nor-adrenaline) and cortisol from the head kidney and enables the individual to cope with the stressor and maintain homeostasis (138). Hypoxia triggers a stress response in teleost fish (139). The stress response increases the cardiac output, gill perfusion, Hb-O_2 affinity, erythrocyte count by splenic contraction, blood glucose level due to release from the glycogen stores and reduces appetite, all of which improves the ability to handle hypoxia (140). Increased blood catecholamine levels were detected in rainbow trout exposed to acute hypoxia and was suggested to cause an increase in the Hb-O_2 affinity (141,142). Atlantic salmon exposed to acute hypoxia responded by increased level of plasma cortisol in a cyclic hypoxia trial (12). However, no increase in plasma cortisol was observed after the first hypoxic episodes during that study (12), which may suggests an acclimation response to the cyclic hypoxia. In rainbow trout exposed to hypoxia (35 or 50 % oxygen saturation for 72 hours), an increase of plasma cortisol was detected, however, plasma lactate was only elevated in fish exposed to 35 % O_2 saturation (121). Futhermore, during strenuous exercise or handling stress, rainbow trout increased the oxygen consumption rate due to increased RMR (143), which may affect the hypoxia tolerance of the fish.

Cardiac responses to hypoxia

Bradycardia, i.e. a reduction of the contraction rate of the heart, is a common response to hypoxia in teleost fish (144). The beneficial effect of bradycardia during hypoxic periods is an increase in the oxygen uptake of the myocardium due to increased ventricular filling and a longer residual time of the blood in the ventricular lumen (144). However, the stroke volume during hypoxia-induced bradycardia is increased and thus maintain the cardiac output until a critical O_2 level (145). Speers-Roesch et al (2010) reported a reduced cardiac power output (CPO) due to bradycardia induced by severe hypoxia (5 % of air saturation at 22 °C for 8 hours) in the hypoxia-tolerant tilapia (*Oreochromis* hybrid sp.) (146). The reduction in CPO reduced the cardiac ATP demand and turnover and hence, the anaerobic glycolysis was sufficient to meet the energy demand of the heart. The researchers also observed a rapid restoration of the cardiac parameters within one hour after returning to normoxic conditions, which indicated no irreversible cardiomyocyte damage due to the hypoxic exposure (146). In a study on *in situ* perfused hearts from a hypoxia-tolerant strain of rainbow trout, a reduced maximal cardiac performance subsequent to exposure of anoxia (20 minutes at 10 °C or 15 °C) was detected (147). In another study, the ATP concentration ([ATP]) in the heart was significantly reduced during hypoxia in rainbow trout. Futhermore, the [ATP] in the brain and red skeletal muscle

remained stable after hypoxic exposure, which suggest that anaerobic respiration occurs is higher in the heart during hypoxia compared to in brain and skeletal muscle (124).

1.5.3   Hypoxia tolerance in salmonids

The hypoxia tolerance of teleost fish is determined by the ability to compensate, both behavioral and physiologically, for environmental hypoxia to maintain or adjust the RMR and to sustain the SMR. The level of dissolved oxygen at which the metabolic rate of the fish is restricted, is termed the limiting oxygen level (LOL). As long as the LOL is above the O_{2crit} needed to sustain SMR, the compensatory mechanisms of the fish (ex. reduced activity, feeding and increased ventilation) sustain aerobic metabolism (67). However, an increased SMR due to increased water temperature, also raises the O_{2crit} (148) and thus lowers the hypoxia tolerance of the fish. Hence, an increased LOL, i.e. a reduced aerobic scope, due to a high SMR or RMR and thus high oxygen consumption rate at the onset of hypoxia, makes the fish less hypoxia tolerant. If the oxygen level continues to decline to the point of O_{2crit}, i.e. the aerobic scope is zero, the fish soon lose the ability to maintain equilibrium. Furthermore, the survival of the fish at oxygen levels below O_{2crit} is determined by the duration of the hypoxia, how far below O_{2crit} the oxygen level stabilizes and the amount of glycogen available for anaerobic fermentation (67).

As for other teleost fish species, the hypoxia tolerance of salmonids can be determined by measuring the O_{2crit} (also called P_{crit} when the oxygen tension is measured) or by registering the level of oxygen at which the fish no longer is capable of maintaining equilibrium (termed loss of equilibrium (LOE)) (149–152). The time until LOE and the corresponding oxygen saturation level is termed the incipient lethal oxygen saturation (ILOS) and is used as a measure for hypoxia tolerance in teleost fish (153,154).

In salmonids, a high variation in the hypoxia tolerance between individuals and strains have been detected (154–156). For Atlantic salmon, dissolved oxygen below 6 mg/L or 60 % O_2 saturation (at 16 °C) are considering hypoxic in the sense that it affects feeding and growth (12,104). However, the threshold level for hypoxia tolerance in Atlantic salmon, when measured as P_{crit}, have been detected to be 3.4 mg/L (at 14 and 18 °C), 4.6 mg/L (at 22 °C) in 12-hour fasted parr (151) and 30 or 55 % O_2 saturation (at 6 or 18 °C, respectively) in non-fasted post-smolts (150). The limiting oxygen level in Atlantic salmon parr (fasted for 24 hours) was found to be 6 mg/L at 12.5 °C in a growth hormone (GH) transgenic group and 4 mg/L in

the control group, suggesting a higher metabolic rate in the transgenic group. Nevertheless, both the transgenic and control group started to loose equilibrium at around 2 mg/L in the same study (149).

The oxygen level that causes Atlantic salmon to loose equilibrium have shown to be lower than the P_{crit} (149,151). The threshold level of oxygen at which Atlantic salmon loose equilibrium have been shown to be 2.19 mg/L DO (151). The time to LOE in 50 % of a fast growing group was significantly higher (260 minutes) compared to a slow growing group (200 minutes) of rainbow trout. The ILOS levels ranged between 13.4 – 16.7 % and 14.7 – 18.9 % of air saturation in the fast and slow growing strains, respectively (154). Zhang and co-workers compared the ILOS (at 12 °C) between a domestic and wild strain of Atlantic salmon parr and detected a higher ILOS in a domesticated strain compared to a wild strain (ILOS = 15.9 % and 14.0 % of air saturation), respectively. Notably, this data was collected by performing a single-fish intermittent-flow respirometry after training the salmon (155).

Several authors have discussed the effect of fish size on the individual hypoxia tolerance. Both an increase and decrease in body mass has been shown to improve the hypoxia tolerance in fish (120,126,157,158). A higher metabolic rate and lower glycogen storage is suggested to reduce the hypoxia tolerance in smaller individuals compared to larger fish (126). In rainbow trout, body mass explained some of the individual variation in hypoxia tolerance, however, no firm association was detected (154). Futhermore, the life stage of the fish may also affect the hypoxia tolerance (126).

To the author's knowledge, the effect on hypoxia tolerance in virus-infected salmonids have not been reported earlier. However, in ISAV infected Atlantic salmon, an impaired cardiac function was observed *in vitro* (159).

1.6 Antiviral immune responses

1.6.1 Components of the immune system

The immune system in vertebrates is divided into an innate and adaptive part. The innate immune system detects pathogens by conserved, germline-encoded receptors, and responds quickly and broadly to intruding pathogens. The adaptive immune system generates antigen-specific receptors by gene rearrangement following the first encounter with a pathogen, leading to immunological memory and a more efficient response to a secondary challenge (160). The memory-based recognition of pathogens is a hallmark of the adaptive immune system, and form the basis for vaccination. The innate and adaptive immune responses interact in many ways, leading to an optimal immune response (161,162).

The evolution of the innate immune system has been fueled by the constant interaction with infectious agents (163,164). Most components of the innate immune system are highly conserved from invertebrates to vertebrates (163,165), demonstrating the importance of the innate immune response throughout evolution. The adaptive immune system evolved in the jawed vertebrates, and cartilaginous fish is the most primitive vertebrate group where immunoglobulins, T cell receptors and major histocompatibility complex (MHC) molecules are present (163,166). There are large differences in the organization of the immune system between the classes of vertebrates. For instance, fish do not have bone marrow or lymph nodes (167,168), and lack immunoglobulin heavy chain class shift (169). Even within fish species there are large differences in the immune system (170). For example, the Atlantic cod (*Gadus morhua*) lack MHC class II and T helper cells, which are important components in the activation of the adaptive immune response (171).

Both the innate and adaptive immune systems consist of humoral and cellular components. The humoral components are proteins with antimicrobial properties and the cellular components are specialized immune cells. Furthermore, the skin, mucus and microflora on mucosal membranes can be regarded as parts of the immune system as they represent a physical barrier against pathogens and possess antimicrobial properties (172). Furthermore, all cells have the ability to respond to intracellular infections and present foreign antigens to the adaptive immune system via MHC class I. In this context, the focus is set on the specific responses raised against viral infections. A short summary of the main components of the innate and adaptive immune system is given in the following sections.

The innate immune system

Macrophages are found resident in most tissues, patrolling for intruding pathogens. They are both present at steady state, and can be recruited and differentiated from monocytes upon infection (173,174). Macrophages are specialized phagocytic cells that engulf pathogens, and performs tissue repair by removing dead cells. The macrophage detects pathogens by recognizing conserved pathogen molecular patterns through specific receptors located in the cell membrane or in the phagosomes (see section 1.6.3 for more details on pathogen recognition). Following phagocytosis, the pathogens are degraded and antigens (normally short peptides) are presented for cells of the adaptive immune system via MHC class II molecules on the cell surface (173). By presenting antigens, macrophages play an important role in connecting the innate and adaptive immune systems. When macrophages are activated by the recognition of pathogens they produce and secrete proinflammatory cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α), chemokines like interleukin 8 (IL-8/CXCL8) that attract neutrophils, and nitric oxide (NO) (175,176). In this manner, the macrophage can recruit and activate the players needed to mobilize an effective immune response against specific pathogens. Teleost macrophages act in the same manner as the mammalian counterparts (177). In salmonids, macrophages have shown to be activated by bacterial stimulation (178–180) and during virus infection by infectious salmon anemia virus (ISAV) (181), SAV (182) and in viral hemorrhagic septicemia virus (VHSV) challenged macrophage/monocyte cells *in vitro* (183). Futhermore, rainbow trout macrophages responded differently when stimulated by a bacterial antigen compared to the synthetic dsRNA molecule polyinosinic–polycytidylic acid (polyI:C), which mimics a viral infection (184).

Melanomacrophages represent a subpopulation of macrophages in ectothermic vertebrates that contain melanin granules and are primarily present in the spleen, kidney and inflamed tissues in teleost fish (185,186). Melanin is a strong antioxidant, and is suggested to protect against the free oxidative radicals released during inflammation (186). In salmonids, melanomacrophages accumulates at sites of long-term antigen retention (186–188). Melanomacrophage centers in the spleen suggest that erythrocytes, pathogens, infected cells or dead cells are removed from the circulation and degraded in this organ (167,168,186). Phagocytosed erythrocytes were detected in both macrophages and melanomacrophages in the spleen during experimental ISAV infection in Atlantic salmon (181).

Neutrophil granulocytes are phagocytic cells characterized by a multi-lobular nucleus, which act primarily against bacterial infections. Neutrophils are among the first immune cells recruited to the site of an infection by chemokines released from activated macrophages or by activated factors of the complement system (189). Their primary function is to destroy pathogens by phagocytosis, reactive oxygen species (ROS) or by releasing toxic granules or neutrophil extracellular traps (NETS) (190,191). The majority of teleost neutrophils are released from the head kidney and constitute less than 5 % of the circulating leucocytes in teleost fish (192). This is in contrast to mammals, in which neutrophils constitutes the majority of the circulating leucocytes (193). Studies on salmonids infected with infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV) and VHSV suggests a role of neutrophils at the site of virus infection (194–196).

Natural killer (NK) cells are lymphoid cells that are considered part of the innate immune system, with the capability of detecting and killing virus-infected cells early after infection. NK cells circulate in the blood and are activated by IL-12 from macrophages or by type II IFN (IFN-γ) produced by virus-infected cells (197). The Fas receptor on the surface of NK cells bind virus-infected cells by attaching to the Fas ligand (FasL) exposed on their surface. The activated NK cell kill the virus-infected cell by releasing perforin that creates pores in the target cell membrane and by injecting granzyme, which induce apoptosis (198,199). Furthermore, NK cells secrete cytokines such as IFN-γ and TNF-α, which activates other cells of the innate and adaptive immune system (197). NK-like cells (also called nonspecific cytotoxic cells (NCCs)) have been described in several teleost fish species including rainbow trout (200). NCCs are suggested to play a role during VHSV-infection in rainbow trout (201,202).

The complement system consists of proteins with antibacterial properties, which also enhances anti-microbial functions of other humoral and cellular components of the immune system. In mammals, this system consists of over 30 proteins, which are primarily found in serum and mucus (203). The complement system is conserved among invertebrates and vertebrates (204). In teleost fish, the complement system is shown to possess similar functions as in mammals. However, the teleost complement system functions at lower temperatures compared to the mammalian (205). Transcription of complement genes in rainbow trout have been detected in the liver, head kidney, intestine, gills, skin, and gonads (206). The role of complement during virus infection in salmonids is not clear as serum neutralization of some viruses seems to be complement dependent and some independent (207,208). An upregulation of C3aR gene

transcripts was detected in the head kidney of IHNV challenged rainbow trout (209). Furthermore, in SAV infected Atlantic salmon, a significant upregulation of complement was detected 16 days post infection (182).

Antimicrobial peptides (AMPs) are evolutionary conserved small proteins common in plants, invertebrates and vertebrates, that act against bacteria, viruses, protozoa and fungi (210,211). Over 2500 AMPs are described in mammals (212) and the AMPs are produced by epithelial cells, neutrophils, monocytes, macrophages, NK cells and in B and T lymphocytes (213,214). Upon receptor-binding of a pathogen, these cells respond by synthesizing AMPs. The AMPs can have several anti-microbial properties; (1) they can form pores in the cell membrane of the microbe, (2) act as chemoattractants for leukocytes, (3) enhance the antimicrobial functions of leukocytes, or (4) affect pro-inflammatory responses (212,214).

The AMPs primarily described in teleost fish are grouped into four families; β-defensins, cathelicidins, hepcidins and the fish-specific piscidin family (215). The AMP genes in teleost fish are primarily expressed in mucosal tissues, i.e. skin, gills and intestine, but are also expressed in liver, kidney and spleen (215,216). In addition to the anti-microbial functions of AMPs, an immunomodulatory function of AMPs in teleost fish has also been suggested (217,218). Piscidins are mainly anti-bacterial, however, anti-viral effects have also been detected (215). The gene expression of β-defensins and hepcidins are upregulated in response to bacterial and viral infection in fish (216). In rainbow trout, upregulation of β-defensin genes in head kidney leucocytes was detected after stimulation by polyI:C (219). Furthermore, anti-viral effects of β-defensin was detected *in vitro* against VHSV (220). Hepcidin genes were upregulated in a rainbow trout macrophage culture subsequent to stimulation with VHSV or polyI:C, and rainbow trout injected with polyI:C also showed increased expression of hepcidin (221). Cathelicidins have mainly anti-bacterial properties (216) and were upregulated in Atlantic salmon and Atlantic cod cell cultures in response to bacterial challenge (222). However, when challenged by polyI:C, only the Atlantic cod cells responded by increased hepcidin gene expression (222).

Cytokines

Cytokines are small, extracellular proteins that act as signal transducers between cells during the immune response. They are secreted subsequent to the sensing of intruding pathogens primarily by macrophages, but other cells also produce cytokines. Cytokines regulate the

inflammatory response and function as chemoattractants (chemokines) (223,224). The pro-inflammatory cytokines that have been best described in teleost fish are TNF-α, IL-1β and IL-6 (225,226). The chemokine CXCL8 attracts neutrophils and macrophages to the site of infection in teleost fish (227,228). Increased gene expression of pro-inflammatory cytokines and IL-8 have been described in salmonids infected with SAV, PRV, IPNV and VHSV (45,229–232).

Interferons (IFNs) are another family of cytokines, which forms the backbone of the antiviral innate immune response. The interferons and their antiviral effects are further described in section 1.6.3.

The adaptive immune system

T lymphocytes are functionally grouped into cytotoxic T cells (CTLs) or helper T cells (Th cells). T cells express a membrane T cell receptor (TCR) which binds to antigens presented on the MHC molecule on the surface of cells. MHC molecules are divided into class I and class II molecules, which in general presents antigens from either intracellular (MHC class I) or extracellular (MHC class II) pathogens. The former is expressed on most infected cells, whereas the latter is expressed primarily on macrophages. T cells express either CD8 or CD4 molecules on their surface membranes, which act as co-receptors for the binding between a TCR and a MHC molecule and are necessary for activation of the T cell. The CD8$^+$ T cells binds to MHC class I molecules and differentiates into CTLs and the CD4$^+$ T cells binds to MHC class II molecules and differentiates into T helper cells (233). In addition, the binding between the T cell signal molecule CD3 and either CD28 or CTLA-4 (cytotoxic T-lymphocyte antigen 4) on the infected cell is necessary for activation of the T cell (234). The activated Th cells secrete cytokines (ex. IL-2, IFN-γ, IL-10, IL-4 and TNF), which enhance the function of B cells, CTLs, macrophages and non-immune cells (235). The activated CTLs have the ability to detect and kill virus-infected cells by perforin/granzyme-mediated mechanisms (199).

In teleost fish, both CTLs and Th cells have been described (236) and are primarily found in the thymus, spleen, peripheral blood and head kidney (237,238). During viral infections, the cytotoxic responses against infected cells are an important part of the adaptive antiviral immune response in teleost fish (239). T cells are also found in the mucosal membranes of teleost fish (172,237), which suggest a role of T cells in the mucosal immunity. Both CD4$^+$ and CD8$^+$ T cells have been identified in salmonids (240,241).

B cells are an important part of the adaptive immune system and their primary function is to produce immunoglobulins (antibodies). B cells express B cell receptors (BCRs), which are membrane bound immunoglobulins with an enormous random variety in their antigen-binding domains. Upon antigen binding, the B cell is activated to divide and expand its population, and differentiate into plasma cells and memory B cells. The memory B cell circulate in blood for long-term memory of the specific antigen, and the plasma cells can secrete large amounts of the antigen-specific antibodies into the circulation. In addition to antigen binding, the activation of B cell requires T helper cells and stimulation by cytokines (242).

The immature B cells of teleost fish are detected in the spleen and head kidney, whereas the maturation and differentiation of B cells subsequent to detection of an antigen, probably takes place in the head kidney (243) (see section 1.6.4 for B cell responses in salmonids during virus infection). Up to the detection of phagocytic teleost B cells in 2006 (244), phagocytosis was thought to be restricted to professional phagocytes, i.e. monocytes, macrophages and neutrophils. Later studies have confirmed the presence of phagocytic B cells in teleost, and also in amphibians, reptiles and mammals (245–248), which outlines the evolutionary development of the B cells (249).

Mature and activated B cells differentiate into plasma cells that produce immunoglobulins. The immunoglobulins are an extremely variable group of small glycoproteins that are found free in serum or mucus, or as membrane bound receptors on B cells (250). Antibodies can exert specific antiviral activity by directly neutralizing the virus or by assisting receptor mediated uptake of virus into macrophages. There are large variations between fish species regarding the production of immunoglobulins. The codfishes lack the MHC class II (171) and do not respond by producing specific antibodies. On the other hand, these fishes have high levels of circulating natural antibodies (251). Furthermore, teleost fish do not possess Ig class switching, and thus have a lower variability in the immunoglobulins they can synthesize compared to mammals (252). The immunoglobulin isotypes described in salmonids are IgM, IgD and IgT (253,254). IgM is the primary immunoglobulin in salmonids and exist both as a membrane bound form or as a secreted tetramer in serum or mucus. The function of secreted IgD in salmonids is not clarified, however, a role in the immune response in the gills have been suggested (255). The tetrameric form of IgT is the most abundant immunoglobulin in mucosal tissues in teleost fish and is believed to have an important anti-microbial role in the mucosal immunity (256–258).

1.6.2   Organization of the immune system in teleost fish

Immunological organs

Teleost fish lack bone marrow and lymph nodes, which are important immunological organs in mammals (259). Instead, the primary lymphoid organs in teleost fish are the head kidney (the anterior kidney or pronephros), thymus and spleen. However, lymphoid aggregates are also present in the mucosa-associated lymphoid tissues (MALT) (167,172,260).

The function of the head kidney substitutes that of the bone marrow in mammals and have been shown to be an important hematopoietic organ in addition to serving immunological functions like B lymphocyte maturation and production of immunoglobulins (167,243). T cells and antigen-presenting cells (MHC class II positive cells) are found in the head kidney of salmonids (261,262), indicating a function of the head kidney in initiating an adaptive immune response. The thymus is located dorsally in the gill chamber, mainly contains T cells (99), and T cell development and maturation occurs in the thymus (236). The teleost spleen function as a secondary lymphoid organ and as a storage for erythrocytes. B cells are numerous in the spleen and the maturation to plasma cells may occur in the spleen (243,261). Furthermore, T cells and melanomacrophage centers are also detected in the spleen (167,237).

The mucosal immune system

In order to establish an infection within the host, the virus needs to pass through the mucosal barriers of the fish. The mucosal membranes in teleost fish cover the nasopharynx, skin, gills and gastrointestinal tract and functions as a first line of defense (172). Therefore, an intact outer mucosal membrane and a functional mucosal immune response is important to stop virus infections. The following section will give an overview of this important part of the immune system of the teleost fish.

The teleost mucosal membranes consist of an outer mucus layer, the epithelial layer and the dermis in the skin or lamina propria in the gills and intestine. The teleost skin also contains scales covered by epithelium that is attached in the dermis and serves as an additional physical barrier (99). The mucus is produced by the goblet cells within the epithelium and contains mainly the glycoprotein mucin and water (263). Immunoglobulins (Ig), complement, lysozyme and antimicrobial peptides are also present in the mucus layer and these proteins initiate the first response to invading viruses of the mucosal membranes (264).

The primary function of the non-keratinized epithelial cells is to form a barrier layer to the outside and in this way protecting the underlying tissue. The epithelial cells also senses viruses and bacteria through pathogen recognition receptors (PRRs) (the molecular basis of virus sensing is provided in section 1.6.3). The intestinal epithelial cells can also absorb macromolecules by endocytosis and present the antigens to macrophages in the lamina propria (265). Macrophages, monocytes, mast cells (tissue resident leukocytes containing AMPs) and dendritic-like cells are embedded in the epithelium and in the dermis of the skin and lamina propria of the gill and intestine (264). Subsequent to an encounter by a pathogen, these cells initiates their antimicrobial responses. The MALT, which consists of lymphoid cells embedded in the mucosal epithelial membranes, has been described in the gills, skin, intestinal wall and the nasopharynx of teleost fish. The MALT contains B and T cells along with components of the innate immune system that respond to infecting pathogens and function as a secondary lymphoid organ where lymphocytes are activated (172). In teleost fish, the MALT have been described in several mucosal tissues and consists of gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT, also called inter-branchial lymphoid tissue (ILT)) and nasopharynx-associated lymphoid tissue (NALT) (172). The most abundant lymphocyte in the mucosal membranes is the T cell and both CD4$^+$ and CD8$^+$ T cells are present (237,238,261,266). The B cells are also detected within the epithelium and in the dermis of the skin or lamina propria of the mucosal membrane in the gills (261) and intestine in teleost fish (267). Both IgT and IgM are detected in the mucus and in the epithelial layer (268), however, IgT is considered the most important player in the teleost mucosal immune response (258). Initial analysis of the cells present in the ILT suggested a presence of MHC class II positive cells, i.e. macrophages and/or T cells (269). Furthermore, aggregates of CD3ε^+ T cells were detected in the ILT (237), which suggests the ILT to have an important role in the antiviral immune response in the gills of Atlantic salmon.

1.6.3 The innate immune response to viral infection in teleosts

The innate immune response is a crucial part of the antiviral defense. Some viruses infect and replicate within the epithelial cells, while other viruses pass the epithelial barrier and enters the blood stream to infect other organs.

Pathogen receptors

The innate immune system recognizes a distinct set of molecules specific for bacteria, virus, parasites or fungi called pathogen-associated molecular patterns (PAMPs). Viral PAMPs are primarily conserved parts of the viruses like nucleic acids (dsRNA, single stranded (ss) RNA or DNA). Double stranded RNA is a potent viral PAMP because it triggers a strong antiviral innate immune response and is produced during the replication cycle of all RNA viruses, either as a dsRNA viral genome, a replicate intermediate or as a product of convergent transcription (270). Long dsRNA molecules are not naturally present in the cell, which enables the innate immune system to interpret dsRNA as a virus specific antigen (271).

The innate immune system detects viral dsRNA by the germline-encoded pathogen recognition receptors (PRRs), which includes the RIG-1 like receptors (RLRs) and toll-like receptor (TLR) 3 (272). The PRRs are evolutionary conserved receptors detected in invertebrates and vertebrates (163), and are either cytosolic (RLRs) or transmembrane (TLRs). RLRs and TLRs are the main receptors detecting viral PAMPs and subsequently initiate transcription of type I and type II interferon genes in addition to genes of proinflammatory cytokines and chemokines (271).

In mammals, the RLRs that detects dsRNA are; retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LPG2) (272). These RLRs are also identified in teleost fish (273), however, the two former are best described in salmonids and the coming section will therefore focus on these. Cytosolic viral dsRNA binds to RIG-I and MDA5, which then phosphorylates the transcription factors interferon regulatory factor 3 (IRF-3) and nuclear factor κβ (NF-κβ). The phosphorylated transcription factors translocate into the nucleus and initiates transcription of type I interferon genes, and genes of pro-inflammatory cytokines (IL-12, IL-6, TNF-α and chemokines) (271).

The toll-like receptors TLR3 and TLR7 are located in the endosome membranes and detect dsRNA and ssRNA, respectively, that is internalized by endocytosis. When bound to dsRNA, TLR3 dimerizes and signals through the adaptor molecule Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF), which initiates phosphorylation of IRF-3 and NF-κβ and the subsequent nuclear translocation and target gene transcription as for RLRs (274). In teleost fish, the TLRs that primarily recognizes viral PAMPs are TLR3 and the teleost specific

TLR22, which both binds dsRNA, and TLR7 that bind ssRNA (275). The TLR22 is located in the cell membrane and detects extracellular dsRNA (276)

The scavenger receptors (SRs) are a diverse family of membrane receptor that bind extracellular PAMPs including dsRNA. By internalizing dsRNA, the SRs expose the viral PAMPs to RLRs and TLRs that triggers an antiviral response (277). This function has so far only described in mammals, however, SR genes are expressed in teleost fish and are suggested to have similar properties (278,279).

Interferons

Interferons (IFNs) have a fundamental role in the innate antiviral immune response in jawed vertebrates. Isaacs and Lindenmann first described interferon in chick embryos responding to influenza virus infection (280). Interferons have been preserved through the extensive evolutionary changes in the teleost genome since the branching off from the common phylogenetic tree that later evolved into amphibians, reptiles, birds and mammals (281). The four introns in the fish IFN genes and the intronless type I IFN genes in tetrapods, suggests a common evolutionary origin of the IFN genes (281).

The IFNs are grouped into type I, II and III based on the receptors they bind. Type I and III (also called IFN-λ) IFNs induce strong antiviral responses (282,283). Type II IFN (also called IFN-γ) are produced mainly by activated NK cells and T helper cells and have a role in both the innate and adaptive immune response against intracellular infections (284).

Type I and II IFNs are described in teleost fish, but type III IFN remains to be identified (285). The type I IFNs are further divided into two groups, i.e. group I and II, based on their number of cysteine bridges (286). Group I type I IFNs are found in all teleost fish and consist of IFN-a, -d and –e, while group II IFNs consist of IFN-b, -c and –f and are only found in certain species of teleost fish (287,288). So far, type II IFNs are only described in salmonids, cyprinid and siluriform species (226).

The type I IFNs described in salmonids are IFN-a -b, -c, –d, -e and -f (288,289) and are produced in nearly all cell types subsequent to virus detection. In Atlantic salmon infected by IPNV, IFN–a1 and IFN-c initiated the strongest upregulation of antiviral genes, while IFN-b triggered a low response and no antiviral response was detected from IFN-d (290). Rainbow

trout IgM$^+$ cells collected from the spleen and peripheral blood were challenged by either VHSV or polyI:C and showed an increased expression of type I IFN genes (291). Gene expression analysis in mature salmonid erythrocytes challenged by ISAV, PRV or polyI:C detected increased transcription of IFN genes, suggesting an antiviral innate immune response mounted by the erythrocytes (16–19,44). Atlantic salmon receiving an intra muscular (i.m.) injection with plasmids expressing IFN-c and -b induced expression of IFN-stimulated genes (ISGs) in head kidney, liver and heart, in contrast to plasmid expressing IFN-a1 that only induced ISG expression locally at the injection site (292). Furthermore, the fish that received IFN-c expressing plasmids were protected against ISAV challenge for eight weeks post IFN-injection (292). Similarly, systemic protection against SAV3 was induced by i.m. injection of IFN-c or IFN-b plasmids in Atlantic salmon, while IFN-a induced only a minor an antiviral response in pancreas and liver (293).

The antiviral effect of IFN-γ in salmonids is not clear and both strong and minor protective effect have been observed against SAV (294,295). In SAV challenged salmon cells (TO cells, i.e. a salmon macrophage cell line), an increased IFN-a gene expression was detected along with increased expression of ISGs. In the same study, only a minor antiviral effect was triggered by IFN-γ (295). Another research group detected a protective antiviral response from both IFN-a and IFN-γ in IPNV or SAV challenged TO cells (294), suggesting a direct antiviral role of IFN-γ.

The antiviral actions of IFNs are mediated through the interferon receptor and the Janus kinase – signal transduction and activator of transcription (JAK-STAT) signaling pathway. The JAK-STAT pathway is a conserved signaling pathway among vertebrates and is triggered by cytokines such as IFNs, interleukins or growth factors. Thus, depending on the type of cytokine, the JAK-STAT pathway may induce antiviral defense, cellular differentiation, proliferation, apoptosis or erythropoiesis (296).

The transmembrane interferon receptors bind extracellular IFNs and activates the intracellular JAK proteins. Depending on the type of IFN that bind the interferon receptor, specific JAK proteins are activated and attracts and phosphorylates the STAT proteins. The dimerized STATs translocate into the nucleus and initiates or inhibits the transcription of a multitude of genes (297). The interferon stimulated genes belongs to a family of genes that are expressed after JAK-STAT signaling and exerts the antiviral effects of IFNs. The suppressors of cytokine

signaling (SOCS) proteins represent another family of ISGs that are transcribed during the JAK-STAT signaling pathway. The SOCS proteins are intracellular proteins that convey a negative feedback on the JAK-STAT pathway (298), thereby reducing the duration and magnitude of the IFN response. SOCS genes have been identified in several organs in salmonids along with their negative effect on the type I and type II IFN signaling pathway (299,300). Recently, SOCS-1 was found to enhance SAV3-infection by inhibiting IFN-signaling in a salmonid cell culture (301).

Interferon stimulated genes

The ISGs encode proteins that have antiviral functions and thus execute the antiviral actions of the IFNs. In teleost fish, several ISGs are upregulated during the innate antiviral immune response (Figure 8). The most readily studied ISGs in teleost fish are summarized in the following section. However, a multitude of putative virus induced immune genes have been detected by transcriptome analysis of salmonids (302,303), but their biological effects remains to be elucidated.
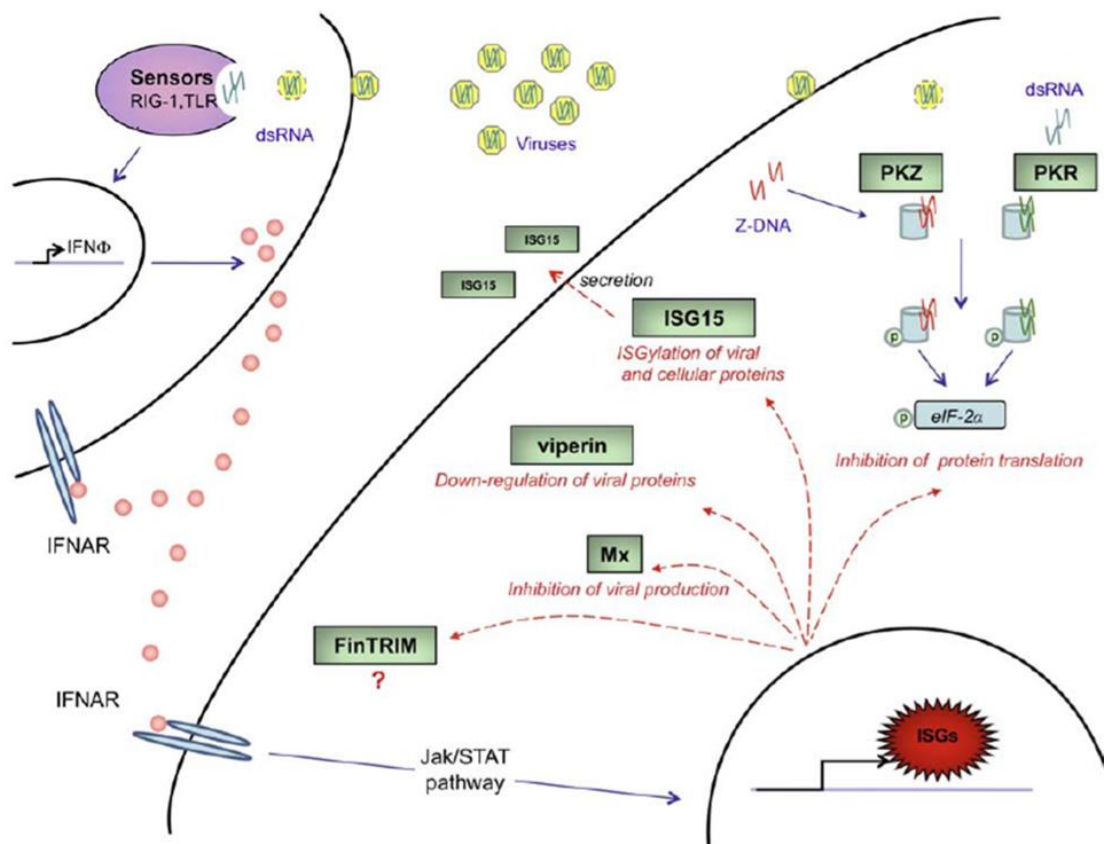
Figure 8. *Synthesis and function of interferon stimulated genes (ISG's) in response to dsRNA in teleost fish. Modified from Verrier et al, Dev. & Comp.Immun, 2011* (302)*, with permission from Elsevier Inc.*

The Myxovirus resistance genes (Mx) are upregulated during virus infection in response to type I and III IFNs and act by blocking early steps of virus replication in the cytoplasm of the cell (304). In salmonids, Mx is shown to be upregulated by several viruses, including the non-enveloped viruses IPNV, PRV and Chum salmon reovirus (CSV) (dsRNA viruses) and the enveloped viruses SAV and ISAV (ssRNA viruses) (17,229,294,305–308).

Another ISG encodes protein kinase R (PKR), which is a protein activated by binding to dsRNA (309). The antiviral function of PKR is through phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2α), which suppresses the cellular protein synthesis and thereby inhibits the translation of the viral mRNA and virus replication (310). PRV infected Atlantic salmon upregulate PKR expression in blood cells (16,17,46).

Vig-1 (VHSV-induced gene) was first described in rainbow trout head kidney leukocytes infected by VHSV (311). Shortly after, vig-1 was renamed Viperin (virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible) after a human homologue expressed in skin culture challenged by inactivated human cytomegalovirus (312). Viperin expression is induced by type I, II and III IFNs during virus infection in mammals and acts on the final stage of the replication of enveloped viruses by inhibiting the budding from the host cell (313). Viperin gene expression is upregulated during PRV and SAV infection in Atlantic salmon (46,306,314). Cholesterol 25-hydroxylase (CH25H) is another ISG that block the fusion of the membranes of enveloped viruses and the host cell, and thereby inhibit virus entry into the cell (315).

The protein Interferon-stimulated gene 15 (ISG15) is an ubiquitin-like molecule that covalently conjugates to other proteins (ISGylation) (316). During virus infection, ISGylation of the transcription factor IRF3 leads to prolonged activation of IRF3 and thus type I IFN gene expression (317). ISG15 transcription is upregulated during ISAV and IPNV infection in Atlantic salmon (318). Furthermore, ISG15 is suggested to have antiviral functions in rainbow trout leukocytes infected with VHSV (319).

Ubiquitin specific peptidase 18 (USP18) is a member of the ubiquitin-specific protease (UBP) family (320) and act as a negative feedback molecule on the antiviral response, in contrast to the other ISGs. USP18 functions as a negative regulator of the IFN production pathways (321) by deISGylation of ISG15 conjugates (322). Furthermore, USP18 prevents IFN signaling by inhibiting the binding of JAK1 to INFAR2 and the subsequent initiation of the JAK-STAT

signaling pathway (323). USP18 was first cloned in teleost fish by Chen and co-workers (2015) and suggested to have similar functions to the mammalian homologues (324).

1.6.4 **The adaptive antiviral immune response in salmonids**

The adaptive antiviral immune response is characterized by an antigen specific response and the generation of immunological memory. The latter is particularly important during a second encounter of a virus and for the development of vaccines. Cytotoxic responses is an important part of the adaptive antiviral immune response in salmonids. In Atlantic salmon infected by ISAV, induced expression of CD8 and MHC class I and II indicating a T cell mediated response (325,326). A T cell mediated immune responses was also suggested in Atlantic salmon infected by PRV or SAV due to an increased expression of CD4 and CD8 (45,229,327). Futhermore, an immune response dominated by CD8$^+$ cells were detected in PRV-infected Atlantic salmon (45). In ISAV challenged Atlantic salmon, an up-regulation of the MHC class I gene was found in spleen and head kidney suggesting initiation of a CTL mediated antiviral response (325).

Immunoglobulins secreted by plasma cells contribute to the humoral antiviral immune response. Clonal expansion of IgM$^+$ and IgT$^+$ B cells in spleen was detected in response to VHSV-infection in rainbow trout (328). Increased expression of type I IFN, TLR3 and MHC class II molecules have been detected in IgM$^+$ cells in spleen after VHSV infection and suggest virus sensing and antigen presentation by these cells (291). The IgM repertoire in Atlantic salmon infected with PMCV was recently characterized by high-throughput sequencing of the immunoglobulin variable region (Ig-seq). The analysis detected an increased transcription of IgM clonotypes which were shared by all infected individuals (329), indicating that certain abundant clonotypes are important for the salmonid B cell response.

1.6.5 **Environmental effects on the immune response**

Farmed fish encounter several environmental stressors that may affect the immune response (330,331). Factors that may stress the fish are water temperature and oxygen content reaching or exceeding critical levels, changes in salinity, predators, strong water currents, crowding and handling (138,330,332–334). In specific, management factors such as sea lice treatments, sorting, and transport, may stress the farmed Atlantic salmon, increase plasma cortisol levels and can have negative impact on the performance of the fish (335), and may increase the

susceptibility to infections. Fish exposed to chronic stress have shown reduced resistance to pathogens (334,336,337). The following section gives an overview on the main environmental stressors experienced by farmed Atlantic salmon and their effects on the immune system.

Environmental hypoxia induce stress responses in teleost fish (332,334). Atlantic salmon exposed to chronic hypoxia showed a lower upregulation of innate antiviral immune genes and cytokines subsequent to *in vivo* or *in vitro* polyI:C stimulation compared to fish reared in normoxic conditions. These results may suggest an impaired antiviral innate response due to chronic hypoxia (13). However, no effect of chronic hypoxia (6.5 − 7.0 mg/L DO) on SAV infection and PD development in Atlantic salmon was detected (338). Futhermore, in Atlantic salmon exposed to chronic hypoxia (50 % dissolved oxygen at 8 and 16 °C), an increased inflammatory response was detected in the intestinal mucosa. The response was most severe at the highest temperature (339).

Rodriguez et al (2016) detected a significantly reduction in the gene expression levels of IFN-$\alpha 1$ and Mx in Atlantic salmon exposed to one episode of acute hypoxia (30% O_2 saturation, 90 minutes, 12 °C) (340). Atlantic cod (*Gadus morhua*) exposed to a six weeks period of continuous hypoxia (46 % O_2 saturation, 8.5 °C) showed a significantly down-regulation of genes related to antioxidant defense and a stress protein in the liver, suggesting reduced protein synthesis during hypoxia (341). These findings may indicate a decreased antiviral responsiveness due to hypoxia.

Crowding stress and handling of fish elicits a stress response that may affect the immune response of the fish (334). Seawater reared farmed Atlantic salmon experience crowding stress during de-louse treatments and during sorting and transport of the fish. Several studies investigating effects of crowding or handling stress in salmonids have detected increased levels of cortisol in blood, reduced growth or body mass and reduced immune responses (342–347).

The majority of teleost fish are ectothermic and their body temperature will therefore vary with the ambient water temperature. Most physiological processes in fish, including the immune response, are affected by temperature and may thus be influenced by the ambient water temperature (348). Both high and low water temperatures may alter the gene expression profiles of several immune related genes in salmonids. Sockeye salmon reared at 8 °C or 12 °C mounted

a better innate response at the lower temperature subsequent to a bacterial challenge. However, a higher antibody response was detected in serum at 12 °C compared to at 8 °C (349). Analysis of the skin of Atlantic salmon reared at 4 °C, 10 °C or 16 °C showed an increased expression of the genes of heat shock proteins, chemokines, lectins and TNF in the individuals kept at 4°C. These findings suggests an increased mucosal immune responsiveness at colder temperatures in Atlantic salmon (350). A transcriptome analysis on erythrocytes collected from rainbow trout exposed to an episode of heat stress (1 hour of 25 °C) showed increased transcription levels of MCH class I, Viperin and genes related to regulation of apoptosis, inflammation and hematopoiesis (351).

Fever is a common response during virus infections in mammals, however, not possible in the ectothermic teleost fish. Interestingly, virus-infected zebrafish increased their survival by moving to higher temperature waters, suggesting a behavioral induced fever (352).

1.7 Effects of PRV infection in Atlantic salmon

1.7.1 Hypoxic stress during PRV infection

Environmental hypoxia in farmed Atlantic salmon may be a source of acute or chronic stress and may thus alter the immune response, the susceptibility for infection, and the disease development in the fish. Virus infections targeting the salmonid heart and erythrocytes, being central in the cardiorespiratory circuit, may also affect the cardiac performance and the tolerance to critical oxygen levels.

As PRV infects over 50 % of the Atlantic salmon erythrocytes at the peak of the infection (15) and the infected erythrocytes mounts an antiviral innate immune response (16,17), the translational and transcriptional machineries of the cells are likely to be affected. This may potentially affect the Hb concentration and the oxygen-carrying capacity of the erythrocytes. During hypoxia in teleost fish, the number of circulating immature and mature erythrocytes increases due to splenic contraction (acute response) and erythropoiesis (subacute-chronic response) (353). This may represent an increase in PRV-susceptible erythrocytes and hence, hypoxic episodes may affect the PRV infection. Since hypoxia affects the immune response, the inflammation caused by PRV infection may also be altered by hypoxic conditions and thus, the development of HSMI.

1.7.2 **The host response to PRV infection**

The reovirus replication cycle is displayed in Figure 9 and a short summary is given. The virus particle enters the cell by receptor-mediated endocytosis, in which subsequent partial capsid disassembly forms the infectious subvirion particle (ISVP). The ISVP penetrates the endosomal membrane. This penetration comes with further disassembly and a non-infectious viral core particle enter the cell cytoplasm. Transcription of virus genes takes place within the core particles and viral mRNAs are released into the cytoplasm. This is followed by the synthesis of virus proteins by utilization of the cellular translational machinery. The non-structural viral proteins forms viral factories, which are enclosures in which new reovirus capsids are assembled. Synthesis of minus strand RNA, forming the dsRNA genome, occurs within the capsid. Finally, the outer part of the capsid is assembled and the newly formed virus particles are released from the host cell (354).
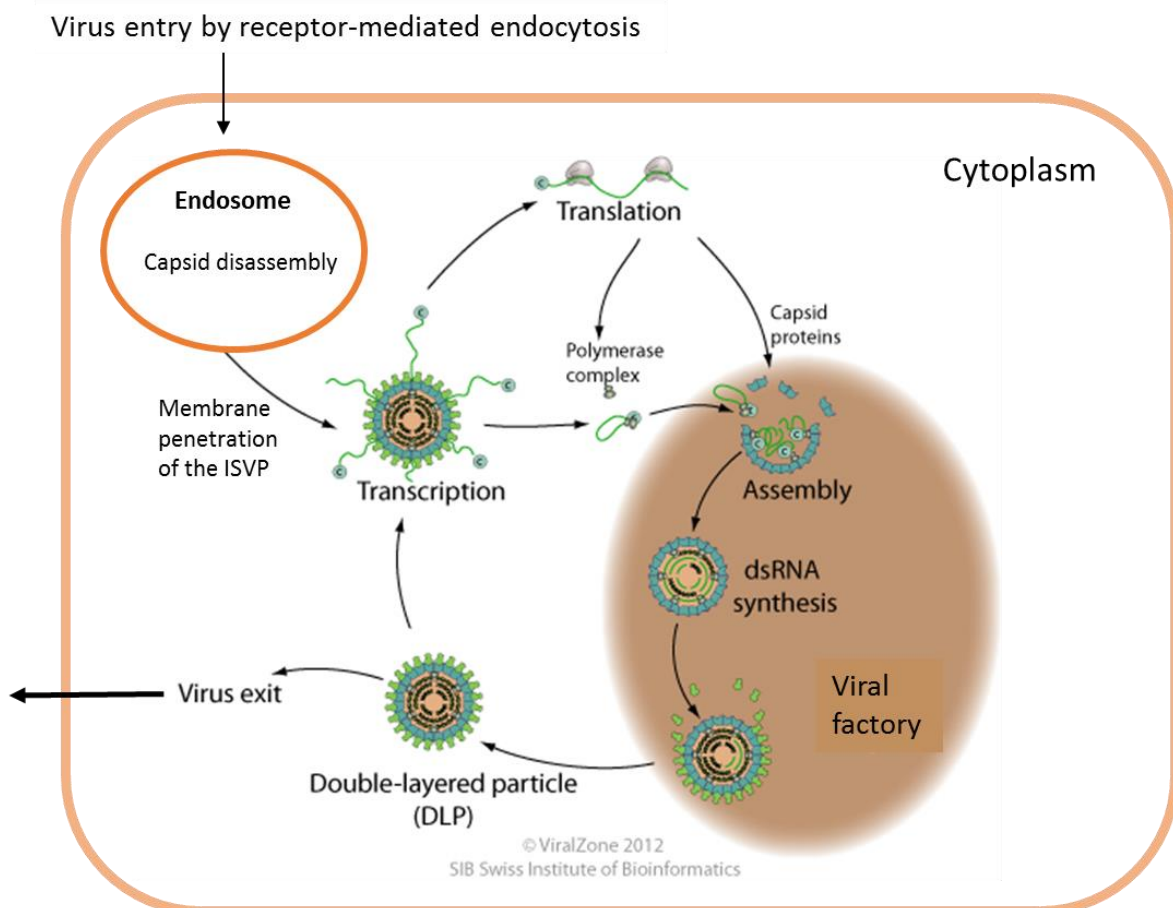
Figure 9. *Reovirus replication cycle. ISVP: infectious subvirion particle. Modified from ViralZone. Source: ViralZone:www.expasy.org/viralzone, SIB Swiss Institute of Bioinformatics.*

The segmented dsRNA genome of PRV is a strong inducer of the innate antiviral immune system in Atlantic salmon. To minimalize exposure of the dsRNA virus genome, the reovirus capsid is never fully decomposed during the virus life cycle. Transcription of the incoming virus genome occurs within subviral core particles where the outer layer has been decomposed in the penetration process, while synthesis of new dsRNA genomes occurs within newly made capsids (354). Furthermore, the synthesized viral mRNA produce proteins that makes secluded structures in the cytoplasm, called virus factories, under the directory of the viral μNS protein (355). Within these viral factories, virus genome replication and construction of new virus particles occur (355). Theoretically, in a successful replication process, the PRV genome should not be exposed and therefore not induce an antiviral immune response. However, potential exposure of PRV dsRNA to the host cell initiates the IFN gene expression and JAK-STAT signaling. Gene expression analysis of the innate antiviral immune response in PRV infected Atlantic salmon shows upregulation of type I and II INF genes, RIG-1, JAK and STAT genes (17). Downstream upregulation of Mx, PKR, Viperin, ISG15, IL-10, IL-10R and IL-12 have been detected up to 10 weeks post PRV challenge (16,17,45,46). Microarray analysis of heart, spleen and head kidney from PRV infected Atlantic salmon show an up-regulation of antiviral innate immune genes and genes related to the B and T cell immune response (356,357). This could indicate species differences in the response or differences between PRV strains regarding induction of immune genes. Futhermore, the detection of CD8^{+} T cell infiltration in the myocardium in addition to increased expression of CD4 and CD8α genes indicates an T cell mediated response in HSMI diseased fish (45). The antiviral innate immune response mounted by the erythrocytes in PRV infected Atlantic salmon (16,17) is likely to act systemically.

Contrary to the findings in Norwegian Atlantic salmon, no strong antiviral immune response was detected in PRV infected Atlantic salmon or Sockeye salmon in BC Canada (48,358). Furthermore, the researchers did not find histopathological changes resembling HSMI in the infected fish (48). However, the timing of the testing of Atlantic salmon after PRV infection was different in the Canadian and the Norwegian studies, and the genera *Salmo* and *Oncorhynchus* have been divided for many millions of years. This may point out a virulence difference between the different PRV subtypes present in the populations of salmon in BC Canada and Norway and/or a difference in host susceptibility for PRV. Furthermore, mammalian reovirus strains have different ability to induce IFN expression, which thus may affect the immune response of the host (359).

PRV proteins in blood cells are only detectable for a 2 – 3 week period 4 – 6 weeks after cohabitation exposure, i.e. approximately 2 - 4 weeks after infection in Atlantic salmon pre-smolts (46). During the initial stage of infection, the researchers detected a strong correlation with PRV RNA Ct values and virus proteins. However, 2 weeks after virus proteins appear in blood cells, they are no longer detectable, despite high PRV RNA levels 6 weeks post challenge (WPC) (46). These results suggest that virus transcription continues while virus protein synthesis is severely reduced, tentatively because of the innate immune response. This suggest that the immune response restrain the production of new virus particles, but that viral transcription and/or replication continue, and the infection is not cleared from the fish.

PRV is a RNA virus and since RNA is not a stable molecule within a cell, RNA viruses, as opposed to DNA viruses, cannot cause true latent infections where the virus genome is dormant. PRV RNA has been detected by RT-qPCR several months after a field outbreak of HSMI (14, Paper III) and for approximately a year in experimental infections (48). In the experimental infection, these long term infected fish could not infect naïve fish that was added to the tanks (48). It is not known if persistently infected fish under stressing field conditions such as a delousing treatment can shed virus. Nevertheless, persistent PRV infection may be activated during stress, affect the physiology of the fish, impair the hypoxia tolerance; which may be detrimental for the fish when handled or stressed.

2   Aims of the study

The overall aim was to study the interaction between PRV infection and secondary stressors in Atlantic salmon with a focus on physiological and immunological effects.

Sub-goals

1. Determine the effects of PRV infection and HSMI on hypoxia tolerance of Atlantic salmon (Paper I)

2. Clarify the effects of transient hypoxic stress on PRV infection and development of HSMI (Paper I)

3. Study the effects of PRV infection on the maximum heart rate and the oxygen-carrying capacity of erythrocytes (Paper I)

4. Characterize the effects of a primary PRV infection on a secondary SAV infection and development of pancreas disease (Paper II)

5. Study the immunological mechanisms involved in a PRV-SAV co-infection (Paper III)

6. Determine if the outcome of a secondary SAV infection after a primary PRV infection is influenced by the SAV subtype (Paper II and III)

3 Summary of papers

Paper I

Hypoxia tolerance and responses to hypoxic stress during heart and skeletal muscle inflammation in Atlantic salmon (*Salmo salar*)

In this study, we aimed to investigate the effects of a PRV infection on hypoxia tolerance, cardiac performance and blood oxygen transport in Atlantic salmon. The current study also investigated the effects of transient hypoxia on the PRV infected salmon. A cohabitation trial with PRV-infected post-smolts was performed. One infected group was exposed to periodic hypoxic stress (4 h of 40 % O_2; PRV-H group) at 4, 7 and 10 weeks post-infection (WPI), and one infected group was reared under normoxic conditions (PRV group), along with one non-infected control group. A standardized hypoxia challenge test (HCT) was used to determine differences in hypoxia tolerance. Both PRV-infected groups showed significantly reduced hypoxia tolerance at 7 WPI when PRV RNA levels peaked in blood. Reduced hypoxia tolerance and cardiac performance was also found in the PRV-infected group at 10 WPI, i.e. at peak levels of pathological changes in the heart. The PRV-H group performed equal to non-infected fish, implying a positive effect of the transient hypoxic stress episodes. However, the PRV-H group had reduced hemoglobin-oxygen affinity compared to non-infected fish. The transient hypoxic episodes did not alter the development of HSMI. In conclusion, PRV-infected Atlantic salmon su have reduced hypoxia tolerance and cardiac performance, which can be improved by pre-conditioning the fish to hypoxia.

Paper II

Experimental *Piscine orthoreovirus* infection mediates protection against pancreas disease in Atlantic salmon (*Salmo salar*)

This study aimed to investigate the interactions between PRV and another important virus in Norwegian aquaculture, salmonid alphavirus (SAV), in a co-infection trial. The effect of a primary PRV infection on subsequent SAV infection was investigated. PRV and SAV are often present in the same locations and co-infections occur, but the effect of this crosstalk on disease development has not been investigated. In this study, Atlantic salmon were infected with PRV by cohabitation, followed by addition of SAV shedder fish 4 or 10 weeks after the initial PRV infection. Histopathological evaluation, monitoring of viral RNA levels and host gene expression analysis were used to assess disease development. Both PRV and SAV target heart and skeletal muscles, but SAV additionally targets the exocrine pancreas. Significant reduction of SAV RNA levels and of PD specific histopathological changes were observed in the co-infected groups compared to fish infected by SAV only. A strong correlation was found between histopathological development and expression of disease related genes in heart. In conclusion, experimentally PRV infected salmon are less susceptible to secondary SAV infection and development of PD.

Paper III

Immunological interactions between *Piscine orthoreovirus* and *Salmonid alphavirus* infections in Atlantic salmon

In this study, the immunological mechanisms involved in the PRV-mediated protection against pancreas disease observed in Paper II were investigated. The expression profiles of a panel of innate antiviral response genes and the plasma SAV neutralization titers were examined. The innate antiviral response genes were in general upregulated for at least ten weeks after the primary PRV infection. Plasma from co-infected fish had lower SAV neutralizing titers compared to the controls infected with SAV only, in line with lower infection levels. Plasma from some individuals infected with PRV only neutralized SAV, but complement inactivation by heat treatment abolished this effect in all but one sample. Analysis of data from a longitudinal field study of a PRV-SAV co-infected fish population indicated a negative correlation between the two viruses in randomly sampled apparently healthy fish, which was in line with the

experimental findings. These results indicate that the long-lasing innate antiviral response induced by PRV is the primary mechanism of protection against a secondary SAV infection.

4   Methodological considerations

Most data from Papers I – III originate from two large PRV cohabitation challenge trials in Atlantic salmon. In the hypoxia-challenge trial (Paper I), physiological measurements and related tests constituted the bulk of the methods, whereas for the co-infection challenge trial (Paper II and III) transcriptional analysis and several immunological methods were used. The following section contain some considerations of the methods used. A more detailed description of the materials and methods is given in the papers.

4.1   Experimental virus infections in Atlantic salmon

In vivo experimental models are commonly used when studying virus infections in Atlantic salmon. The challenge trials presented in Paper I and II were performed at the indoor aquatic research facility of VESO Vikan, Namsos, Norway. By performing controlled indoor experimental challenges, the environmental parameters like water temperature, oxygen saturation, light regime and salinity can be determined and monitored throughout the trial. This minimizes the influence of potential unknown factors on the experimental fish during the challenge trial. Examples on different challenge models used for studies of the most common virus diseases in Atlantic salmon are given in Table 3.

	I.p. or i.m. inoculation	Cohabitation challenge	Bath/immersion challenge
Salmonid alphavirus (SAV)	Andersen et al (2010) (338)	Taksdal et al (2014) (360)	Jarungsriapisit et al, (2016) (361)
Infectious salmon anemia virus (ISAV)	Caruffo et al (2016): i.p. (362)	Mikalsen et al (2001) (363)	Aamelfot et al (2015) (364)
Infectious pancreas necrosis virus (IPNV)			Robledo et al (2016) (365)
Piscine orthoreovirus (PRV)	Kongtorp et al (2009): i.p.(56) Mikalsen et al (2012): i.m. (45)	Finstad et al (2014) (15)	
Piscine myocarditis virus (PMCV)	Timmerhaus et al (2012): i.p. (366) Martinez-Rubio et al (2014): i.m. (367)		

Table 3. *Table showing different challenge models used to induce experimental infection with common viruses of Atlantic salmon.*

The main routes of virus challenge in Atlantic salmon are: 1. By injection either intraperitoneally (i.p.) or intramuscularly (i.m.) with virus-containing cell culture medium or tissue homogenate; 2. Cohabitant challenge with shedder fish; 3. Bath or immersion challenge. The i.p. or i.m. infection models are useful when knowledge about the investigated disease and/or the pathogen is scarce or if exact virus dose and time of infection is essential to control. The two latter models aims to mimic a natural infection by exposing the naïve fish to the virus through the water and not by physically depositing the virus into the fish. In the cohabitant model, the naïve fish are kept in the same tank as the virus shedders, i.e. with fish that have been previously infected with the virus. The ratio between shedders and naïve fish may range between 1:25 and 1:1 (15,360,363), depending on the type of virus, transmission efficiency, the virus level in the shedders and the purpose of the trial. In challenge trials investigating the pathogenesis of a virus and transmission efficiency, a lower ratio may be sufficient, while a higher ratio to ensure a homogenous and synchronous infection is warranted when studying secondary responses to the infection, like in both of our challenge studies. A natural infection route, i.e. cohabitation, and natural responses to infection, are also crucial in our studies. A problem concerning the cohabitation model is the variation in onset of infection of the

cohabitants. This may result in individual differences in the time course of infection, the level of virus RNA and corresponding immune responses. This larger variation requires larger experimental groups to avoid that the differences blur the interpretation of the data.

Bath immersion challenge has also been used to mimic a natural virus infection in Atlantic salmon (361,363–365). The bath immersion model can be performed by adding *in vitro* cultured virus to the tank holding the naïve fish (363–365), or by adding the naïve fish into the tank containing virus produced by shedders which have been removed (361). In the latter method, the virus titer is determined by analyzing tank water samples. By using the bath immersion model, one can gain information about the time of onset of infection and dose of virus exposed to of the naïve fish. However, a short exposure to high titer of virus does not necessarily mimic the natural virus exposure. To the author's knowledge, a bath immersion model have not been developed for PRV infection and was thus not considered used for the challenge trials in this thesis.

The PRV challenge model

The PRV challenge model used in Paper I and II was cohabitation in a 1:1 shedder: cohabitant ratio. The shedder fish had been inoculated i.p. with blood cell lysates from PRV infected fish. Due to the lack of a functional *in vitro* culturing system, the common challenge material used for PRV infection is virus-infected blood cells collected during the peak phase of PRV replication in blood. The lack of an *in vitro* culturing system also makes it impossible to measure the amount of infective virus in the inoculum. Therefore, the level of PRV RNA, i.e. Ct value of an RT-qPCR assay targeting PRV, is used to estimate the virus load. The challenge material should contain a maximum amount of infective virus, and the time-point for collection of material is crucial to obtain high infectivity. In our experience, blood cells harvested early at the peak of PRV RNA level in blood is the best material (15). In the initial phase of replication in blood, there is a better correlation between the amount of infective virus and viral RNA (PRV Ct value), but this correlation is lost after the peak of infection in blood cells, most likely due to a halt in translation in the infected cells (46). The preparation of the inoculum and quantification method of PRV RNA is described in detail in the papers (Paper I - II). Tissue homogenates from either heart, spleen, plasma, liver or head kidney collected from infected fish, have also been used as challenge material in previous studies (15,56,356). However, due

to lower amount of infective virus in these tissues compared to blood cells, such challenge material was not used for our challenge trials.

The cohabitation challenge model could have been improved if a standardized amount of infectious virus had been used to inoculate the shedders, which would have reduced the variability between experiments. VESO Vikan minimize the variation between experiments by using blood from the same original batch for their PRV challenge experiments and use standardized procedures for inoculation securing equal amount of inoculum injected into each fish. Recently infective PRV in high concentrations have been obtained by virus purification from PRV-infected blood (Wessel et al, submitted PLoS ONE, 2017). This allow copy number calculation and thus infection with a specific numbers of PRV particles, but exact measures of infectivity can still not be obtained.

The challenge trial described in Paper I was performed on seawater-adapted post-smolts reared in brackish water (salinity of 25 ‰). Post-smolts were selected in this study because PRV is most prevalent in seawater and environmental hypoxia is most commonly encountered at sea. The reduced salinity of the tank water was selected to minimize the risk of bacterial skin infections in lacerations, since the plan was to increase fish density during the hypoxic episodes in one of the groups. In Paper I, the weeks post introduction of PRV shedders into the tank containing naïve fish are addressed as weeks post infection (WPI) and not weeks post challenge (WPC) to avoid confusion with the "challenge" by physiological test. This is, of course not completely correct, as the time of exposure to PRV, i.e. introduction of PRV shedders, does not necessarily equals time of infection for the cohabitants.

In the co-infection trial in Paper II and III, seawater adapted post-smolts reared in seawater (salinity of 32 ‰) was used to resemble a field setting during the grow-out phase of Atlantic salmon. The farmed Atlantic salmon is likely to be infected by PRV shortly after transfer to seawater (14). PRV infection have also been detected in fresh-water facilities (6,14), which increase the possibilities of smolts being PRV-infected already at seawater transfer. In contrast, all outbreaks of PD and detections of SAV have been observed in the marine phase of Norwegian salmon farming. Thus, we wanted to study a primary PRV-infected salmon population, which was secondary challenged by SAV. The co-infection challenge trial presented in Paper II consisted of 4 or 10 weeks of PRV infection (WPC-PRV) followed by a six weeks long period of PRV-SAV co-infection. The different time-points for SAV-shedder

introduction (i.e. 4 and 10 WPC-PRV) aimed to introduce the SAV co-challenge during increasing PRV replication (4 WPC-PRV) or at peak cardiac inflammation (10 WPC-PRV) (Paper II). The details of the co-infection challenge trial is illustrated in Figure 16 in the Results and general discussion section.

Considerations related to the three R's (reduction, replacement, refinement)

Before performing experiments on experimental animals, any researcher needs to consider how the concept of the three R's can be implemented in the experimental design according to Russel and Bruch (1959) (368). The aim is to minimize the number of experimental animals used in research and to optimize the welfare of the experimental animals. The three R's includes; (1) Reduction: reduce the number of experimental animals used, (2) Replacement: replace experimental animals with alternative methods, (3) Refinement: optimizing the welfare of the experimental animals throughout the study (369).

"Refinement" implies the use of methods that reduce the pain and stress on the experimental fish during the trial, like the use of sedatives and anesthesia before handling and sampling (369). Using methods which minimize the sensation of pain in research animals is mandatory by law (370) Minimizing stress and handling throughout the trial and ensuring regular husbandry routines, also improve animal welfare. Good fish welfare is obtained by ensuring sable and optimal water quality (ex. oxygen content and water flow) and fish density (371). To improve the fish welfare in the experiments performed in Paper I, the experimental fish were euthanized as an end-point after the hypoxia challenge test (HCT), instead of returning the fish to the mother tank. Futhermore, the fish density was not significantly increased during the HCT and the transient hypoxic episodes. During the temperature-dependent maximum heart rate measurements, the fish were sedated during the experiments, thus reducing the stress on the fish.

"Replacement" warrant the researcher to consider alternative methods that can be used instead of experimental animals to answer the study aims. Such methods can be computer models and cell or tissue cultures (372). Several different cell cultures are used in fish research to study immune responses, infection kinetics and stress responses (373). Due to the physiological tests performed in Paper I and the lack of a functional cell culturing system for PRV, no alternative methods were available for studying PRV-infection.

"Reduction" implies the use of a minimum of experimental animals, which is achieved by calculating the necessary sample size and by considering alternative methods, both of which still ensures results that are statistically valid (374). The sample size is determined by the probability of detecting a significant result if the difference between the experimental groups are real, i.e. the power of the test (375).

In the challenge trials in Paper I and II, the number of experimental fish could have been reduced by lowering the number of shedder fish. However, to obtain an optimal transmission efficiency of PRV to the naïve fish, a 1:1 shedder:cohabitant ratio was necessary. The number of experimental animals in a challenge trial can also be reduced by lowering the number of sampling time-points. In the experiments performed in Paper I and II, the number of sampling time-points was carefully selected to be able to investigate the strategically important phases of the PRV-infection. Non-lethal sampling is another method that may reduce the sample size and have been performed to obtain muscle tissue (376), gill tissue (377), blood (378) and liver samples (379) from fish. Mucus is also possible to sample without sacrificing the fish (380). However, non-lethal sampling of tissue or blood requires physical handling of the fish and potential induction of pain or infection at the sampling site. Hence, the welfare of the experimental fish needs to be considered before performing non-lethal sampling.

In our experiments, non-lethal sampling of blood could have added useful information. The erythrocytes are major target cells for PRV and blood is therefore suitable for studying PRV infection kinetics. The PRV infected erythrocytes mount a strong antiviral innate immune response, which can be studied by qPCR and/or immunostaining methods in blood. Furthermore, analysis of blood parameters such as Hb, hematocrit, pH and cortisol may add useful information about hematological or stress responses performed in Paper I. Non-lethal sampling of blood have been reported for Atlantic salmon and rainbow trout experimentally infected with ISAV or *Yersinia ruckeri*, respectively (378,381). Furthermore, non-lethal sampling was used to document affinity maturation in rainbow trout during a period of 24 weeks (382). However, non-lethal sampling of peripheral blood is limited to experimental fish having a body mass over 150 - 200 grams (381). Although PRV cohabitation trials are highly reproducible regarding virus levels and the development of histopathological changes, there is still missing information about the pathogenesis of HSMI. In order to investigate the immunological or physiological differences between experimental groups, we needed to sample

vital organs like heart, head kidney and spleen, in addition to blood. The implementation of non-lethal sampling also requires training and validation of the effects of the method on the experimental fish and on the particular disease. Currently, non-lethal sampling has not been reported used in HSMI studies in Atlantic salmon.

4.2   Quantitative PCR

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is the common molecular method for detecting specific RNA sequences. In Paper I, II and III, RT-qPCR was performed to quantify the virus RNA levels and the relative expression of various host response genes during the challenge trials.

The methodological basis of an RT-qPCR reaction consists of three steps; the reverse transcription, amplification and quantification. The first step includes the action of the reverse transcriptase (RT) enzyme, which produces a complementary DNA strand (cDNA) from the RNA. Viruses like PRV have a double stranded RNA genome, which has to be separated into two ssRNA strands before initiating the RT-step. The second step of the RT-qPCR reaction is the amplification of the cDNA by repeated cycles of polymerization and denaturation producing copies of the sequence targeted by specific primers. At the end of each amplification cycle, fluorescence is emitted, either from the probe or from emitters intercalated in the dsDNA amplicons. The fluorescence is quantified, and this reflects the level of dsDNA copies. Usually, 40 - 45 cycles are run in a qPCR. The last step is the relative quantification of the amplification products by defining how many amplification cycles is needed before the fluorescence signal crosses a pre-set threshold. The threshold for the fluorescence signal can be set several standard deviations above the mean fluorescence baseline. The cycle number at which the amplification curve crosses the fluorescence threshold is the cycle threshold (Ct) value. The Ct value is inversely related to the level of the target RNA in the original tissue sample, i.e. the higher the Ct value, the lower level of the target RNA.

In Paper I – III we use two different detection methods: Fluorescent probes are used for detection of viral RNA and a fluorescent intercalating dye (SYBR Green) for host gene analysis. The probe is labelled with a fluorescent dye, which is released in each amplification cycle due to the exonuclease function of the polymerase. The SYBR Green dye binds to all dsDNA molecules and emits a fluorescent signal when intercalated between the base pairs in the double

helix. The SYBR Green detection method is therefore not specific for the target sequence but will also emit fluorescence if bound to erroneous amplicons. An initial test of the assay run on an agarose gel and a melt curve analysis at each RT-qPCR run needs to be performed to confirm that only the correct amplicon is produced.

The high specificity and sensitivity of RT-qPCR is an advantage for detection of low levels of a specific target. However, several factors can influence the outcome; (1) The RNA quality: RNA is easily degraded by RNases, which are present everywhere in our environment as they are natural part of the skin and mucosa. To protect the RNA, the sample needs to be stored in an RNase free environment, in the presence of RNase inhibitors and at low temperatures. (2) The reverse transcription reaction should be 100 % efficient, i.e. all target RNA molecules should be copied into cDNA to allow detection of a single target RNA molecule. Optimal reverse transcription may be disturbed by inhibitory factors in impure samples, and the access of the RT-primers to incompletely denatured target RNA. (3) The qPCR assay design should be optimized by considering amplicon length, primer properties and primer specificity.

Virus detection

In Paper I – III, RT-qPCR was used to determine PRV RNA levels in heart, blood and spleen in the experimental fish. The RT-qPCR assay used targeted the L3 gene segment of PRV (383). The result of an RT-qPCR reaction reflects the relative number of target RNA sequences (see "Gene expression analyses" below). For PRV-RNA detection there are three main targets (1) The dsRNA viral genome which is mainly present within intact virus particles in equimolar proportions, i.e. one copy of each genome segment per virion. (2) The viral mRNA, which cannot be distinguished from the positive strand of the genome since the whole segment length is transcribed. (3) The negative strand of the genome during replication before the complete dsRNA is produced. Depending upon the phase of the viral life cycle, the type of sample collected and the target segment chosen for the PRV PCR, the RT-qPCR result will reflect the dominating target in the sample. For example, an organ sample collected during the active replication phase will mainly reflect the level of viral mRNA, while a cell free serum sample will mainly reflect the dsRNA genome from virus particles.

In Paper II and III, RT-qPCR was used to quantify the level of SAV RNA by targeting the SAV nsP1 gene, which encode a non-structural protein and is thus not a part of the high-copy number sub-genomic RNA encoding the structural protein (384). A PCR targeting the high-copy

number sub-genomic RNA for SAV would probably be more sensitive during the active replication phase of SAV. However, the nsP1 gene is a commonly used target for RT-qPCR. A Ct value of 37.0 was used as a cut-off point in both the PRV and SAV qPCR assays to distinguish false positive from true positive samples. The determination of a cut-off value can be determined by several statistical approaches, which is discussed by (385).

Gene expression analysis

In Paper II and III, RT-qPCR was used to assess expression of a number of host response genes during PRV and SAV infection in Atlantic salmon. Relative quantification of the level of the gene of interest (GOI) can be performed by normalizing the Ct value against at least one stably expressed reference gene (386). Reference genes are chosen based on stable expression level in the target organ and their expression level should not be altered by the conditions tested in a given experiment. Examples of reference genes used in gene expression studies in salmonids are elongation factor 1 α β (EF1αβ), 18S ribosomal RNA, β-actin, structural ribosomal protein S20 (RPS20), acidic ribosomal protein (ARP) or Dynein (358,387). The change in expression level of the GOI can be calculated by the $2^{-\Delta\Delta CT}$ method (388). The $2^{-\Delta\Delta CT}$ method corrects the expression level of the GOI to the expression level of a reference gene in the same sample (ΔCt) and secondly against a defined ΔCt basal value, i.e. from a specific time-point or control group (386). During virus infection in Atlantic salmon, the transcription levels of the most common reference genes are altered in several organs (17,387). However, the gene expression of EF1αβ was stable in virus infected hearts and β-actin was stable in virus-infected erythrocytes (17,387). In Paper III, the gene expression of several innate antiviral immune genes and antimicrobial peptides was investigated by RT-qPCR. In this paper, the relative expression level of the GOI in heart was calculated by the $2^{-\Delta\Delta CT}$ method and EF1αβ was used as a reference gene using the mean expression at Day 0 as the reference time-point. However, in blood and spleen the expression of the reference gene was altered by the infection. Hence, the data generated from blood and spleen was standardized only by using a fixed amount of total RNA input in the qPCR reaction.

All RT-qPCR assays used in the gene expression analysis in Paper II and III had been tested and validated prior to analysis. The assays were tested *in silico* for false targets by BLAST search, and the amplification result was tested by gel electrophoresis to confirm a single qPCR product of the correct length. In addition, most assays were designed to cross exon-intron boundaries to avoid genomic DNA background, and this background was also controlled by

running qPCR on a sample which had not been reverse transcribed. Although RT-qPCR is a sensitive relative quantification method for RNA, the result does not necessarily imply that the corresponding proteins are produced. Thus, the biological significance of an induced gene expression detected by RT-qPCR needs to be confirmed by quantification of the target protein or its activity.

4.3 Histopathological examinations

Histopathological lesions in selected organs from the challenge trials were scored blindly to assess HSMI development in the experimental groups (Paper I and II). The organs were fixed for 24 h in 10 % phosphate buffered formalin when sampled and embedded in paraffin wax before sectioning and subsequent staining with hematoxylin and eosin according to standard procedures.

Histopathological scoring of HSMI

In one of the first studies on experimentally induced HSMI, Kongtorp et al (2009) systematically scored inflammatory changes in the epicardium, endocardium, around cardiac blood vessels, compactum and spongiosum (56). In that study, the researchers determined categorically the presence of inflammatory changes in the heart of the experimental fish. Later, a graded scoring system, either categorical or continuous, have been used in HSMI diseased hearts (45,57,389). Mikalsen et al (2012) evaluated the overall inflammation in the four cardiac compartments, atrium, epicardium, compactum and spongiosum, by using a visual analogue scale (45). In this method, the examinator determine the score by marking the individual scores relative to each other on a visual analog scale, ranging between 0 – 3, based on specific criteria defining the severity of cardiac inflammation and myocardial necrosis (45). Finstad et al (2012) used this scoring system, however the epicardium and myocardium were given separate scores according to the specified scoring criteria for the respective heart compartments (57). In another study, HSMI diseased hearts were evaluated by a categorical semi-quantitative scoring system ranging from 0 to 3 (389). That method was based on the scoring system for histopathological lesions of pancreas disease in Atlantic salmon, developed by McLoughlin (2006) (390) and graded the severity of inflammatory changes into four categories based on specific criteria (389).

The scoring method of choice when assessing the histopathological lesions in HSMI depends on the purpose of the experiment. A generalized categorical scoring of the heart is sufficient if the researcher just needs a confirmation if HSMI is present or not. However, if the disease development between the experimental groups is expected to differ, the inflammatory changes within each of the cardiac compartments should be scored, either by using an ordinal or continuous method. The continuous scoring method is time consuming and laborious but allows the use of parametric statistical methods (ex. Student t-test) on the result, which improves the statistical power of the analysis. Furthermore, the continuous scoring gives a better resolution of the histopathological changes in the infected groups, which may enable the researcher to detect important differences. The ordinal categorical system is less time consuming and may reduce the differences in scoring results between examinators and hence, different studies. Analysis of ordinal data requires the usage of non-parametric statistical tests (ex. Mann-Whitney U test), which have a lower statistical power. However, non-parametric tests are often used independently of scoring methods due to a low number of individuals in the experimental groups.

Nevertheless, when assessing the histopathological data in meta-analysis of challenge trials, it is important to have in mind that histopathological scoring is a subjective evaluation.

Histopathological scoring of PD

The scoring of histopathological lesions in Atlantic salmon with pancreas disease is performed on the exocrine pancreas, the heart and skeletal muscle (360,390). The severity of the lesions is scored separately for each organ according to a semi-quantitative system ranging between 0 – 3 (360,390). Histopathological changes in the heart and skeletal muscle are consistent findings in both PD and HSMI (49,391), however, necrosis and loss of exocrine pancreas tissue is only described in fish with pancreas disease (PD) (55) (Table 2).

The histopathological scoring method used in the different papers

A continuous scoring of the inflammatory changes in Paper I was performed according to a visual analogue scale ranging between 0 – 3, modified from Mikalsen et al (2012) and Finstad et al (2012) (45,57). The examined heart compartments, i.e. epicardium, compactum and spongiosum, were scored separately based on the scoring criteria described in Paper I.

The scoring of HSMI related inflammatory changes in the hearts of the experimental groups in Paper II was performed by an ordinal, i.e. categorical, scoring system. The details of the scoring criteria are described in Paper II. The changes related to PD in the SAV infected and PRV-SAV co-infected groups were scored in the exocrine pancreas and myocardium according to the same type of scoring system. Scoring of inflammatory changes and myocardial necrosis was performed in heart, red and white skeletal muscle and pancreas to confirm and separate the development of HSMI and PD in the experimental groups (Paper II). The scoring system was also used to assess differences in disease development in the experimental groups.

4.4 Physiological measurements and tests performed in the hypoxia challenge trial

The challenge trial in Paper I included three experimental groups; a PRV-infected group, a PRV-infected group exposed to transient hypoxic episodes and a non-infected control group. During the challenge trial, various cardiorespiratory tests were performed to investigate the effect of PRV infection on the cardiorespiratory system at three levels; cellular (i.e. the erythrocyte), organ (i.e. the heart) and whole organism (i.e. hypoxia challenge test) in Atlantic salmon. The transient hypoxic episodes were performed after finishing all other tests performed at each time-point.

The acute hypoxia challenge test (HCT)

An acute HCT was performed 4, 7 and 10 weeks post PRV infection to investigate the hypoxia tolerance of the fish in the PRV-infected and non-infected groups.

The acute hypoxia challenge test was performed in a common garden set up in a separate tank (425 liters, called the HCT tank). The test included 30 fish from each experimental group that were transferred to the HCT-tank and was allowed to acclimate overnight before the test. All naïve fish dedicated to be cohabitants were PIT tagged before starting the challenge trial, which enabled determination of tank affiliation. The oxygen saturation in the water was initially reduced by the oxygen consumption of the fish after turning off the water flow into the tank. The oxygen saturation was reduced gradually to 25 % during a time course of 50 - 60 minutes. A further decrease of 2 - 4 % O_2 per hour was achieved by bubbling in nitrogen gas through a gas-mixing column (Figure 10). The oxygen saturation in the tank water was continuously recorded and logged by two fiber optic oxygen meters. The fish started to loose equilibrium as

the O_2 saturation declined. When a fish lost the ability to keep equilibrium, it was immediately removed from the tank, and the group affiliation, time and corresponding oxygen saturation level (i.e. the incipient lethal oxygen saturation (ILOS)) was recorded. When all fish had reached ILOS, the test was terminated.

Figure 10. *The hypoxia challenge test (HCT). Dr. Sven Martin Jørgensen (NOFIMA, currently FHF) tests the recirculation system and mixture of nitrogen gas into the tank water. Photo: Morten Lund*

To the author's knowledge, this is the first reported acute HCT performed on experimentally virus infected Atlantic salmon. Acute HCTs have been performed earlier to determine individual ILOS as a measure for hypoxia tolerance in salmonids and European sea bass (153,154,156,392). Hypoxia tolerance in teleost fish may also be measured by determination of the critical oxygen saturation level by individual respirometry (393). Individual respirometry is highly reproducible, but is time consuming and thus limits the number of replicates per sampling. Due to the need for a large sample size per group to be able to detect a difference in hypoxia tolerance between the experimental groups, the acute HCT were found to be suitable for our study.

The acute HCT is time efficient, non-lethal and allows the researcher to test a large number of fish simultaneously in a common garden setup, i.e. several groups are mixed in the test tank. The problem with tank-to-tank variations is avoided by performing the acute HCT, in addition

to gaining sufficient statistical power. Furthermore, as the HCT is a non-lethal method (153), it allows several HCTs to be performed during a trial if necessary.

During hypoxia, the blood and ambient water accumulate waste products such as CO_2 and ammonia in addition to declined levels of dissolved oxygen. Therefore, the HCT determines both the hypoxia and hypercapnia tolerance of the fish. We did not measure the CO_2 content in the tank water or the blood pH in this study due to practical limitations. Data from these parameters would have added complementary information about the physiological changes in the blood of the fish during the challenge trial. However, the aim of the HCT in our study was to differentiate the hypoxia/hypercapnia tolerance at fish level. Nevertheless, these measurements should be included in future studies. Hypercapnia can be avoided by performing open-flow or intermittent-flow respirometry as the accumulation of CO_2 and ammonia in the ambient water is minimal in these methods (393).

The progressive decline in oxygen saturation induced a stress response in the fish, which triggered the fish to jump, leading to mixing of O_2 into the water. The jumping caused a disturbance in the decline in oxygen levels in our study, and covering of the water surface with a lid or net may have prevented this. The physiological stress response triggered by the hypoxia/hypercapnia and the handling of the fish during the HCT may have influenced the individual performance during the test. Hence, data on parameters such as plasma adrenaline, cortisol or lactate would have made it possible to assess the stress response triggered by the individual fish. However, by euthanizing the fish after reaching ILOS, the stress responses may not have influenced the main outcome parameters of the acute HCT in our study, i.e. the performance on organism level during a HCT in the infected and non-infected groups, the PRV RNA levels in blood and heart and the histopathological changes in heart.

The transient hypoxic exposures

One of the PRV-infected groups in Paper I was exposed to transient episodes of hypoxia at three time-points during the challenge trial. The aim was to investigate if exposure to transient hypoxia affected the virus infection and the development of HSMI. The selected time-points for the hypoxic episodes aimed to expose the infected fish at peak virus replication but prior to heart inflammation (4 WPI); peak virus load and increasing heart inflammation (7 WPI); and at peak heart inflammation (10 WPI). The transient hypoxic episodes were performed after finishing all other tests performed at each time-point.

The transient hypoxia episode was initiated by reducing the tank water supply to a minimum without reducing the water level significantly, and allow the oxygen saturation to progressively decline by the oxygen consumption of the fish and minimal exchange of water. The tank water (12 °C) reached 40 % oxygen saturation approximately 30 minutes after reducing the water flow and was kept at this level for four hours by regulating the water flow into the tank. The fish were continuously monitored visually during the hypoxic episode and the oxygen saturation was registered manually every 15 minutes. At the end of the hypoxic episode, the oxygen level was normalized within 60 minutes by gradually increasing the water inlet. No mortality was registered during or after any of the hypoxic episodes.

The 40 % oxygen saturation used in the transient hypoxic episodes was decided based on previous studies showing that 39 - 40 % oxygen saturation is a limiting oxygen saturation level for Atlantic salmon (149,150). To the authors' knowledge, there are no earlier reports on virus infected Atlantic salmon challenged with hypoxia below 60 % O_2 saturation. Andersen and co-workers detected no effect of continuous hypoxia (60 - 65 % O_2 saturation at 12 °C) on the development of SAV infection in Atlantic salmon (338).

The minimal water flow into the tank and the reduced swimming activity of the fish could have caused unequal levels of oxygen saturation within the tank due to reduced water circulation. However, the oxygen saturation was monitored by two oximeters, and we did not observe large variation. Nevertheless, increased water circulation could have been ensured by adding propeller pumps in the tank to even out possible differences in levels of O_2 and CO_2.

The tank water volume was not reduced during the period of transient hypoxia and the fish density was thus unchanged during the hypoxic episode. This ensured minimum crowding stress on the fish, and the main stress factor was hypoxia/hypercapnia. The stress response to the hypoxia/hypercapnia itself could have been monitored in all experimental groups by measuring the plasma adrenaline, cortisol or lactate. This would have provided important details about the responses during hypoxia and the impact on the subsequent tests performed in the challenge trial. However, the cortisol response in Atlantic salmon exposed to cyclic hypoxia is previously shown to be minimal after the first hypoxic exposure (12), which may suggest a reduced impact of cortisol during hypoxia exposure. Moreover, the transient hypoxic episodes were performed at the end of each sampling time-point, i.e. after finishing the other tests, thus

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minimizing the acute effects of the hypoxic stress on these tests. Nevertheless, measurements of water parameters such as pH and/or CO_2 levels would have added complementary data on the specific environmental conditions experienced by the fish during the transient hypoxia and throughout the challenge trial.

Finally, by including a non-infected control group exposed to transient hypoxic episodes we could have collected additional information when assessing the effects of hypoxic stress on PRV-infected compared to non-infected fish.

Temperature-dependent maximum heart rate measurement
In order to evaluate the effects of PRV infection and HSMI on cardiac performance, individual temperature-dependent maximum heart rate measurements (f_{Hmax}) were performed in Paper I. This method allows the estimation of critical and optimum temperature (T_{opt}) for the cardiac function of individual fish. The measurements were performed on all three experimental groups and at 10 weeks post infection, i.e. at peak levels of inflammatory changes in heart.

The temperature-dependent maximum heart rate measurement was performed as described earlier (394). To summarize the experimental set up, the experimental fish were anesthetized and placed in a custom-made chamber. The gills were flushed with aerated and temperature-controlled water continuously. Electrodes were placed under the fish's heart in contact with the skin and an electrocardiogram (ECG) was recorded (Figure 11A and B). Initially, fish were acclimatized to allow the heart rate to stabilize for 30 minutes at 12 °C before the fish received an intra peritoneal injection of atropine sulfate that blocked the vagal inhibition of the heartbeat. The water temperature was increased by 1 °C every 6 minutes and f_{Hmax} was recorded for each temperature step. The experiment was terminated when reaching 20 °C and before cardiac arrhythmias occurred. The fish were then euthanized and samples collected.
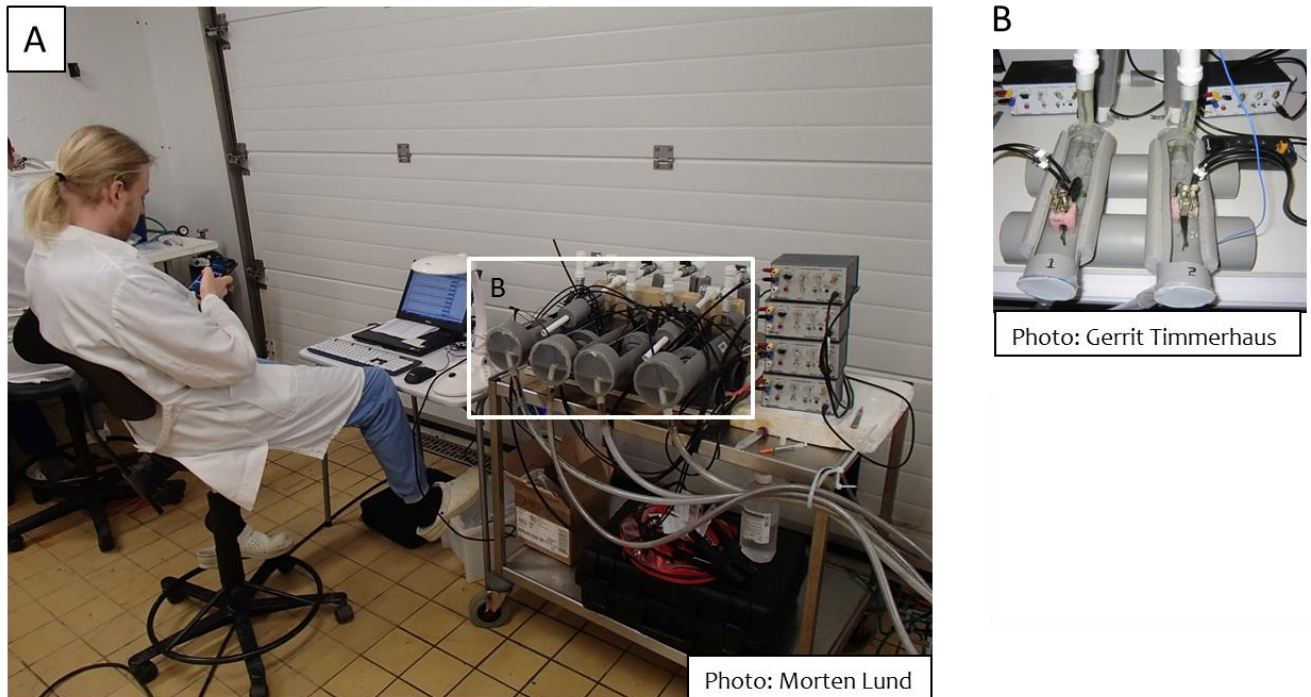
Figure 11. *Temperature-dependent maximum heart rate measurement. A. Dr. Gerrit Timmerhaus (NOFIMA) monitors the heart rate measurements. The experimental fish are kept in one chamber (gray colored) each. Photo: Morten Lund. B. The fish within the test chamber with the electrodes recording the electrocardiogram positioned under the heart Photo: Gerrit Timmerhaus.*

The temperature-dependent maximum heart rate measurements have been performed earlier as a measure for *in vivo* cardiac performance in salmonids (70,394–396). The temperature optimum (T_{opt}) for maximum aerobic scope was calculated in Paper I (see section 4.9 for details on the calculation of T_{opt}). The T_{opt} for maximum aerobic scope is the temperature at which the maximum cardiac output no longer can increase (68). Thus, a lower T_{opt} for maximum aerobic scope indicates a reduced cardiac performance (397), and has been used to monitor cardiac performance between different families of salmonids or between resting and exercised salmonids (69,396).

Maximum heart rate measurements were also performed 4 weeks post infection to assess the cardiac performance at an early stage of PRV infection. These results were imprecise due to electrical interferences in the facility at this particular sampling, and were not used.

The temperature-dependent maximum heart rate measurements is a reproducible method. However, it is time consuming and specialized equipment is needed to perform the experiment.

In our study, measurements of cardiac ventricle mass could have added useful data to the heart rate measurements, as an increased ventricle mass improves the temperature tolerance and cardiac output in salmonids (156,398). Furthermore, cardiorespiratory performance have also been tested by detecting maximum swimming performance in a tunnel respirometer (399,400). However, due to practical limitations this method was not found suitable for the project.

Hemoglobin-oxygen dissociation measurement

In Paper I, we aimed to study the hemoglobin-oxygen affinity of the erythrocytes in the PRV-infected blood, and this was done by performing hemoglobin-oxygen dissociation measurements.

The hemoglobin-oxygen dissociation measurements was analyzed according to the Tucker method (401). Briefly, one experimental fish at a time was anesthetized and peripheral venous blood was collected from the caudal vein in a heparinized tube. The blood sample was added to a rotating tonometer and kept at 12 °C by circulating water around the tonometer. The blood was added propranolol (1.0×10^{-5} M) to block the adrenaline and nor-adrenaline effects on the β-adrenoreceptors, which affects the oxygen binding affinity of hemoglobin in salmonids. The blood was then successively de-saturated by gassing the blood sample with nitrogen. At successive levels of oxygenation determined by the direct measurement of the blood partial pressure (PO_2), the total oxygen content of the blood was determined according to the Tucker method in a Tucker cell. After finishing the measurements, a sample of the blood was collected for hemoglobin and ATP measurements (details on the Hb and ATP measurements are include in Paper I).

The total oxygen content data-points were corrected for Hb concentration after subtracting the physically dissolved O_2 (402), and the Hb-O_2 saturation was calculated. The PO_2 values and the corresponding Hb-O_2 saturation was plotted against each other and a curve, i.e. the oxygen dissociation curve (ODC), was fitted by a local polynomial regression fit (by using a R software package). The P_{50} value, i.e. the PO_2 at which 50 % of the Hb is oxygenated, can be determined from the ODC and is used to describe the oxygen affinity for hemoglobin.

The oxygen-carrying capacity of salmonid blood have been readily studied by collecting oxygen dissociation data and plotting an oxygen dissociation curve (76,130,401,402). By studying differences in the ODC, the researchers can determine factors that affect the

hemoglobin-oxygen affinity, including the Bohr and Root effect. However, this is a time-consuming method that requires experienced personnel with access to specialized equipment and may restrict the use of this method.

In our experiment, we did not measure blood pH, although this could have added valuable information. Furthermore, as we aimed to detect any differences in Hb-O_2 affinity between the infected and non-infected groups, we did not perform the measurements immediately after the hypoxic episodes. Future studies investigating the hypoxia in PRV-infected Atlantic salmon, should include hemoglobin-oxygen dissociation measurements directly after the hypoxic episode, as this may complement the data collected in Paper I. Furthermore, by performing the hemoglobin-oxygen dissociation measurements at an earlier stage of the PRV-infection than what was done in Paper I, one could possibly have detected an impact of peak PRV replication in erythrocytes on the Hb-O_2 affinity.

4.5 Microarray analysis

In Paper II, an oligonucleotide microarray analysis was performed to improve the overall understanding of the host transcriptome responses in heart tissue during the PRV-SAV co-infection. The analysis was performed on hearts from SAV3 infected fish and from PRV-SAV3 co-infected fish , 4 and 6 weeks after SAV3 challenge (both groups).

Oligonucleotide microarrays enable quantitative simultaneous detection of thousands of gene transcripts. Microarrays are normally species specific. Based on selected mRNA sequences, thousands of oligonucleotide DNA sequences, i.e. DNA probes, are designed *in silico*. The DNA probes are synthesized on the surface of the microarray slide at specific positions (called "spots"). In case of Agilent microarrays, the probes have a length of sixty nucleotides. For the labelling process, total RNA is extracted from the tissue sample, and reverse transcription using poly-T primers to transcribe mRNA sequences is performed to produce cDNA. The primers also contain a T7 promoter sequence, which become part of the cDNA. Then, T7 RNA polymerase is used to make cRNA from the cDNA templates and in this process Cy3-coupled cytosine nucleotides are used to label the resulting cRNA molecules with a fluorophore. The labeled cRNA is then transferred to the microarray slide and complementary strands of cRNA and DNA probes hybridize. The hybridization process takes place at 65 °C for 17 hours (overnight). Afterwards, the microarray slide is washed to remove unbound molecules and

finally scanned and the Cy3-specific emission intensity for each spot is calculated. Spot intensities are corrected by background subtraction and each array is normalized according to LOESS (locally weighted scatterplot smoothing) method. Differences in gene expression are estimated by statistical comparison of normalized spot intensities from different experimental groups and time-points. Microarray analysis in fish have been used to study immunology, pathology, effects of nutrition, physiology and reproduction (403).

In Paper II, the analysis was performed by using NOFIMA's Atlantic salmon oligonucleotide microarray Salmon Immunity and Quality (SIQ) 6 produced by Agilent Technologies (Santa Clara, CA, USA). The SIQ-6 platform contains 15 k unique probes and was designed by Krasnov et al. (2011) (404). Hearts from un-infected fish sampled at Day 0 in the trial served as a common reference in all hybridizations. The data from the microarray was analyzed by using the bioinformatics package STARS (404). The SIQ-6 microarray platform has previously been used to study host responses in salmonids during bacterial (405,406) and viral infections (17,303,357,407).

Oligonucleotide microarrays provides an overview of the transcriptome and points to genes of interest to be focused on in downstream studies. Microarray analyses are accurate and reproducible, however it is recommended to verify selected gene expression patterns with qPCR. Analyses that evaluate the presence, abundance and functionality of the downstream proteins (proteomics, antibody-based staining techniques or enzymatic assays) enhance the value of the microarray results.

Designing of microarray platforms requires rich sequence information. Discrimination of paralogs including duplicated genes as well as splice isoforms can be problematic. However, these complications are resolved for species with available genomes including Atlantic salmon. Given relatively high costs of microarray analyses, the numbers of samples are commonly limited. Therefore, selection of experimental groups, time-points and individuals is of crucial importance. In this respect, information from previous studies (e.g. challenge trials) and background knowledge such as analysis of virus level and histopathological changes in the target organ are very useful to guide the selection of experimental groups and individuals.

An alternative to oligonucleotide microarray is RNA sequencing (RNA-seq), which detects and quantifies transcripts by using next-generation sequencing. The main advantage of RNA-seq is

the possibility to perform genotyping and gene expression analyses simultaneously. Compared to an oligonucleotide microarray, RNA-seq may have a broader dynamic range and higher sensitivity. The main advantaged of microarrays are simplicity and high speed of both laboratory work and data analyses, which do not require special facilities. Use of standard pre-annotated platforms greatly facilitates and enhances data mining including meta analyses.

4.6   Immunohistochemistry

Immunohistochemical staining of heart tissue sections was performed in Paper II to detect PRV and SAV antigens. In addition, we wanted to investigate if the two viruses were located in close proximity to each other in a co-infected heart.

Immunohistochemistry (IHC) is a technique that uses specific antibodies (monoclonal (mAb) or polyclonal (pAb)) to detect target proteins in histological sections. Monoclonal antibodies are identical clones that bind to a specific epitope of the target antigen and polyclonal Abs are a mix of antibodies binding to different epitopes on the antigen. Polyclonal Ab samples are produced by immunizing an animal (commonly rabbits) with the target protein, and collecting a crude serum sample. Within the animal, different plasma cell clones produce antibodies against the foreign protein that react to different epitopes on the target. Monoclonal Abs are produced by isolating spleen cells after immunization (usually in mice), and amplify a single plasma cell in culture. The advantages of pAb samples are that they are not affected by small changes in the target epitopes because they recognize several epitopes. Hence, they are more tolerant to alterations in the target protein due to changes in pH and salt concentrations or fixation methods used, and are quicker to produce compared to mAbs. The advantages of mAbs are the specificity to only one epitope, that they are produced by a renewable source and have higher purity compared to pAbs (408), minimizing unspecific background staining. The antibody-antigen complexes can be visualized by several methods to study the distribution and tissue location of particular cells or pathogens. Unspecific binding of the antibodies to non-target proteins is a common problem when performing IHC and may cause background staining. Furthermore, if a peroxidase is used for staining, unspecific staining can be caused by endogenous peroxidases in inflamed tissues that may be present.

Polyclonal antibodies binding the outer capsid proteins σ1 and µ1C of PRV was developed by Finstad and co-workers (2012) by immunization of rabbits with recombinantly produced

proteins. These antibodies were used to confirm PRV infection in cardiomyocytes in HSMI diseased Atlantic salmon (57). Later, a pAb was developed against the non-structural protein μNS of PRV, a protein which is only present in cells during replication of the virus (355), and thus function as a replication marker. IHC using the μNS antibody may therefore be an important tool for studying the pathogenesis of HSMI.

The PRV staining protocol performed in Paper II included the rabbit pAb anti-σ1 (57). The SAV staining protocol was performed using a monoclonal murine anti-E2 antibody (409). The PRV and SAV staining was performed on separate sections. However, the sections were cut subsequent to each other, to allow tissue details to be recognized. This enabled visualization and localization of adjacent SAV-infected and PRV-infected cells.

An advantage of IHC when studying virus infection is the possibility to visualize the distribution of virus proteins in relation to the histopathological changes of interest. However, nonspecific binding of antibodies and background staining needs to be controlled when performing IHC. Hence, proper negative controls such as the use of pre-immunization serum or irrelevant mAbs or pAbs, and omitting the primary antibody, should be performed to test the specificity of the method. The specificity of the antibody should be validated by western blotting (410). Monoclonal Abs that bind to conformational dependent epitopes commonly lose their binding capacity in assays where the proteins are denatured. The specificity of the antibody may be evaluated by using blocking peptides, i.e. the antigen sequence used to produce the antibody, that will inhibit staining if the antibody is specific (410). Furthermore, the antibody dilution, incubation times, and washing procedures of the IHC assay must be optimized and to perform the IHC with a negative control antibody to detect any background staining (411).

4.7   Virus neutralization test

In Paper III, we performed a SAV plasma neutralization test in order to investigate whether PRV infection could induce a humoral response that would neutralize SAV. We aimed to elucidate if the interference between the virus infections could partly be ascribed to humoral response, and used a virus neutralization assay for salmonid alphavirus earlier described by Graham and co-workers (208). The plasma samples used in the virus neutralization (VN) test were sampled at 0, 4, 7 and 10 weeks post PRV challenge.

A virus neutralization (VN) test can detect the presence and titer of neutralizing antibodies in plasma or serum. Neutralization means that the viral infectivity is inhibited, i.e. that the ability to infect live cells is lost. Serial two-folds dilutions of heat-treated plasma sample is incubated with a fixed amount of infectious virus. Heat-treatment will inactivate complement, but should not affect the antibodies themselves. The standard convention is to use 100 tissue culture infective dose/50 (TCID/50) of infective virus per inoculated volume. After incubation of the serum-virus mixture to enable antigen-antibody binding, the mixture is added to the cell culture, usually as two parallels in 96 well plates. The SAV infectivity is evaluated either by observation of a cytopathic effect (CPE), or the cells are fixed and immunostained for detection of virus after a fixed time of incubation (3 days for the SAV VN assay). The immunostaining procedure was used in Paper III, although it was modified compared to the Graham paper by using acetone fixation and immunofluorescence staining for SAV. A SAV specific mAb (2D9, against SPDV strain F93-125) (208). The resulting serum/plasma titer is the highest dilution where either CPE or SAV staining is observed. Serum toxicity of fish serum to cultured cells may be a problem in VN assays, but this is usually solved by diluting the sample.

Virus neutralization is an important host mechanism to block the ability of a virus to infect, and antibody binding to the viral particle is the main mechanism. In addition, fish sera/plasma may contain so far unknown virus neutralizing components other than antibodies in addition to complement that may also influence the outcome of a VN assay. The virus infection is usually blocked by hindrance of viral attachment and entry into the cell. Viruses have a broad specter of mechanisms to counteract the neutralizing actions of antibodies, and a common property of many viruses (including SAV) is glycosylation of the envelope proteins to make them less available for antibody binding. Neutralization tests are commonly used to demonstrate neutralizing activity after a SAV infection (208,360,390,412). The salmonid alphavirus is classified into six subtypes, SAV1 – 6. This classification is based upon nucleotide differences in the non-structural proteins, and the subtypes are therefore not different serotypes and should act similarly in a neutralization test (413).

Salmon serum/plasma antibodies are mainly of the IgM type, which are complement binding antibodies. Therefore, the salmon complement may interfere with the results in a neutralization test and it is necessary to address the complement dependence of the neutralization. When testing the neutralizing activity of serum from fish that had been infected with VHSV or IHNV, the respective antibodies were shown to be complement dependent. Thus, in these VN assays,

the sera need to be decomplemented by heat-treatment followed by adding of a defined amount of complement from a naïve fish (207,414). Salmonid complement can also be inactivated by freezing (− 20 °C) of the serum or plasma (415,416). However, the SAV neutralizing assay seems to be complement-independent and the need for adding complement is not required (208).

The use of virus neutralization tests is declining because they are time consuming and require skilled personnel (207,208,417). However, improvements of the detection of SAV by immunoperoxidase staining test have eased the reading of the assay (208).

4.8 Field data

In Paper III, two collections of field data were included. The data originated from the counties of Nord- and Sør-Trøndelag (approximately mid-part of the western coastline of Norway).

The first data series is referred to as the "Screening data" and were derived from heart samples of salmon collected in 2011 – 2014. The samples were collected at 16 time-points on several seawater production sites from fish post mortem or in moribund condition (N = 307). The tissue samples were preserved on RNA*later*^{TM} and collected through a routine surveillance program by a salmon farming company. RT-qPCR were used to determine PRV and SAV RNA levels and the inclusion criterion in this data set was individuals being positive, i.e. Ct value lower than 37.0, for both PRV and SAV on RT-qPCR.

The second data series is referred to as the "Longitudinal study" and was generated during a longitudinal observational study performed in two net pens at one marine production site located in Nord-Trøndelag county. The smolts in the study pens were put into sea June 2014, and a monthly sampling of 20 randomly selected, clinically healthy fish was pursued. HSMI was diagnosed 5 months before SAV2 was detected at the site, which should be considered when evaluating the consequences of the co-infection. The samples included in this study consisted of heart tissue preserved on RNA*later*^{TM} and RT-qPCR were performed to determine PRV and SAV RNA levels. SAV RNA was first detected in the study pens in June 2015, which subsequently were sampled (N = 20) the following 3, 7 and 11 weeks. These time-points were selected to detect peak level of SAV infection, i.e. 3 weeks after first SAV-detection, and the decline in SAV level thereafter, i.e. 7 and 11 weeks after first detection.

4.9 Statistical methods

Data handling and statistical considerations regarding the histopathological data used in Paper I and II are included in section 4.3.

A p value ≤ 0.05 was considered as statistically significant in all tests.

Paper I

Data handling and statistical analysis in Paper I was performed in R version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria). A conservative non-parametric approach (Mann-Whitney unpaired rank test) was chosen to test for differences in virus Ct levels, ILOS levels, hemoglobin and ATP levels between the experimental groups. This was done by performing a Mann-Whitney unpaired rank test by using the R "stats"-package. The reasoning for using a non-parametric test was due to the lack of normality in the distribution of the data-points and due to the low N in each group per sampling point (418). Differences in Fulton's k-factor (k-factor = body mass in grams/(length in cm)^3 * 100) and body mass (gram) between the groups were tested by performing an unpaired Student's t-test due to the normal distribution of the data-points and the continuous nature of the data-points.

The ILOS levels and the corresponding time until ILOS for each group were plotted in a Kaplan-Meier survival plot and differences between the groups were tested by a Peto & Peto modification of the Gehan-Wilcoxon test, all functions embedded in the additional "survival" package in R. The linear relation between body mass and ILOS levels (in all groups included in the HCTs) and PRV Ct values and Hb concentrations (in pre-test individuals only) was investigated by performing a linear regression analysis and a Pearson correlation analysis (R, "stats" and "Hmisc" packages). The Pearson correlation analysis was chosen due to the continuous data tested.

The temperature-dependent maximum heart rate data was computed in R using the additional packages: RColorBrewer, devtools and AquaR. The normalized, maximum heart rates was plotted against the corresponding temperature and a one-way analysis of variance (ANOVA) was performed to compare the f_{Hmax} between the groups at each temperature increment. The T_{opt} for maximum aerobic scope was estimated by calculating the Arrhenius breakpoint temperature as described in (394).

The O_2 saturation and PO_2 registrations were plotted and an oxygen dissociation curve was fitted through the data points by a local polynomial regression fit (R, "loess" function in the "stats" package). The percent oxygen saturation of Hb was calculated. The data was then log transformed ($log10((O_2/gHb)/(1-(O_2/gHb))$ and $log10$ PO_2) and a linear regression line was fitted through the linearized data. The K_d (zero intercept) and Hill coefficient (n_H) was determined using SigmaPlot 10.0 (Systat Software Inc, London, UK).

Paper II and III

In Paper II and III, the statistical analysis was performed in GraphPad PRISM version 7.0 (GraphPad Software Inc., USA) and in STATA version 13.1 (StataCorp, USA). A conservative non-parametric approach (Mann-Whitney unpaired rank test) was chosen to test for differences in virus Ct levels and gene transcript levels between the experimental groups. The reasoning for using a non-parametric test was due to the lack of normality in the distribution of the data-points and due to the low N in the experimental groups at each sampling point. Differences in Fulton's k-factor and body mass between the groups (Paper II only) were tested by performing an unpaired Student's t-test due to the normal distribution of the continuous data.

The relation between the virus Ct values or gene expression data and histopathological scores was investigated by performing a Spearman's rank correlation analysis. The Spearman's rank correlation analysis is a nonparametric measure of the correlation between the ranked values of two variables and can be used on ordinal variables. This test was thus applied on the ordinal nature of the histopathological scores in Paper II. Furthermore, the correlations between the gene expression data and histopathological scores (Paper II) were corrected for multiple comparisons by Bonferroni-adjusted significant level. In Paper III, a Spearman's rank correlation was used to assess the relation between PRV Ct values and gene transcript levels in blood, spleen and heart. The Spearman's rank correlation was performed on the Ct values due to lack of normal distribution of the Ct values and the low N in the groups.

Field data (Paper III)

Epidemiological analysis of retrospective field data is challenging due to a several unknown environmental and management factors that may influence the results. Furthermore, the number of fish sampled in each pen may influence the statistical power of the analysis. The number of individuals sufficient for detecting a pathogen is determined by the prevalence of the pathogen

within the population and the selected confidence interval (419). Thus, when sampling 30 fish in a population of 100 000 individuals and the assumed prevalence of the virus is 10 %, the probability of detecting one positive fish is 95 %. In the total data set, from which the Screening data was extracted, the prevalence of PRV and SAV was 96 % and 53 % (unpublished data), respectively. Hence, the high prevalence of both viruses in the study area makes the sample size in the Screening data and Longitudinal study acceptable.

In our study, we performed a Spearman's rank correlation analysis on both the compiled data and by each sampling-point in both data sets. This method was selected due to the lack of normality in the distribution of the Ct values in the dataset (unpublished data). A correlation analysis does not necessary reflect causation and should be complemented by a regression analysis to better describe the influence of PRV Ct values on the SAV Ct values. An aspect of the field data is that the observations are clustered within time-points and at locations. This clustering must therefore be accounted for during further analysis. However, the large variation observed in the scatterplots indicates an overall low explanatory value of PRV Ct on SAV Ct-value. Taken together with the lack of information about important factors (i.e. mortality rate, growth rate and water temperature), a further regression analysis on these data was not performed.

5 Results and general discussion

The main question addressed in this project was if PRV infection in Atlantic salmon affects the response to and outcome from stressors like hypoxia or secondary infections. The rationale for this question is the high prevalence of PRV in the marine phase of salmon farming, the massive PRV infection of erythrocytes in the initial phase of the infection and the strong antiviral immune response mounted by the erythrocytes. Furthermore, field observations also suggest that PRV-infected fish may be less capable of managing additional environmental stressors, which affects the outcome of the infection (6). Two experimental challenge trials were planned and conducted specifically to test these hypotheses. The results are discussed in the following paragraphs, with a focus on their importance for Atlantic salmon aquaculture.

5.1 PRV infection compromise hypoxia tolerance in Atlantic salmon

Paper I investigated the hypoxia tolerance of PRV infected fish. The incipient lethal oxygen level (ILOS) of individual fish was determined by performing an acute hypoxia challenge test at 4, 7 and 10 weeks post PRV infection (WPI) (Figure 12). The results showed a significantly reduced hypoxia tolerance in the PRV infected group 7 and 10 WPI. However, at 4 WPI the PRV infected group showed an improved hypoxia tolerance compared to the non-infected group.

At 7 WPI, the high levels of PRV RNA in blood and heart (Figure 13) are likely to explain the reduced hypoxia tolerance in these groups. Furthermore, the hemoglobin (Hb) concentration in the infected fish was significantly lower than in the naïve fish at 7 WPI. The reduced hypoxia tolerance at this time-point could be due to several factors; (1) the immune response during the PRV infection in blood and heart; (2) the reduced Hb level during peak PRV infection in blood; or (3) other effects of the PRV replication in blood and heart.
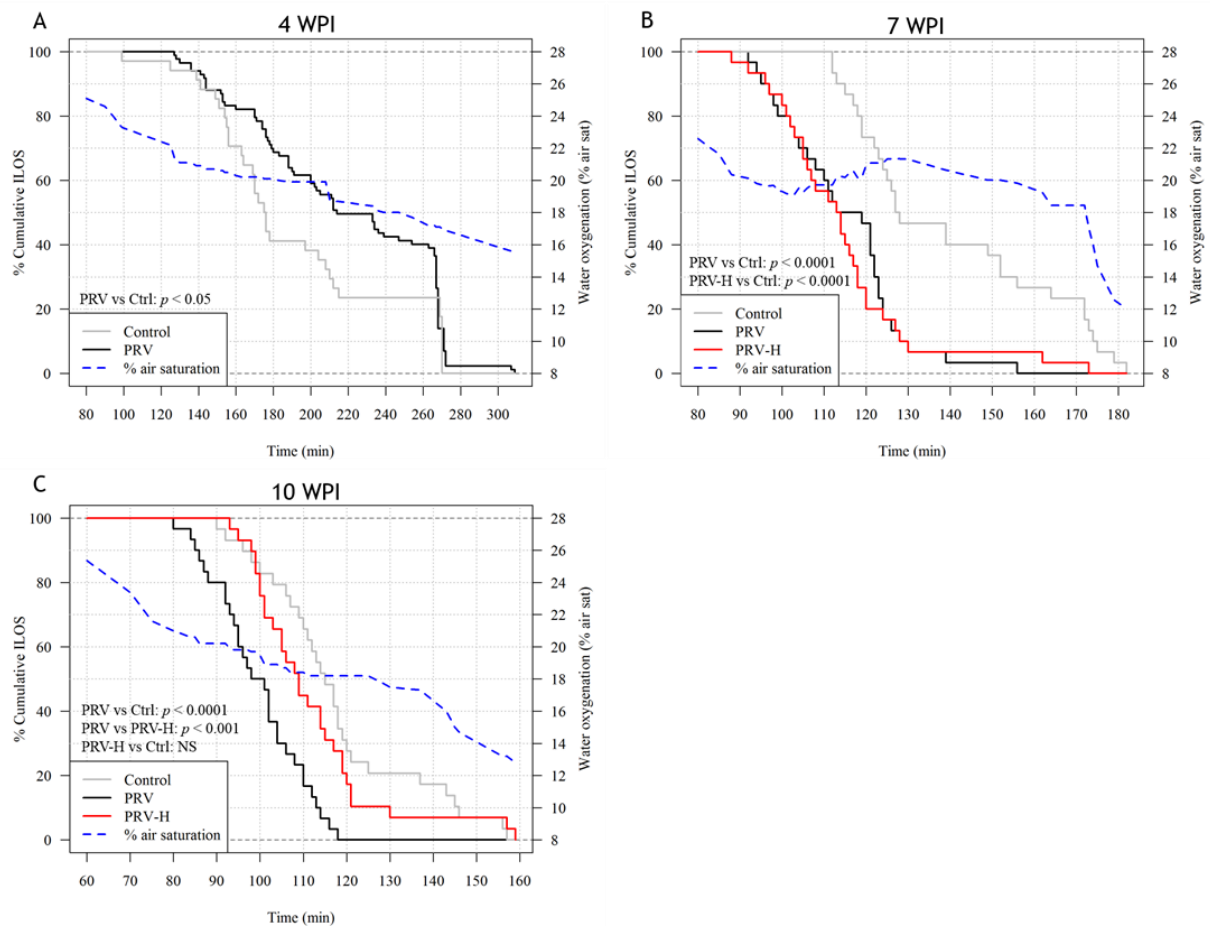
Figure 12. *Kaplan Mayer curves showing the hypoxia tolerance determined by minutes to ILOS. Groups are indicated by Ctrl (grey line), PRV (black line) and PRV-H (red line) at 4 (A), 7 (B) and 10 (C) weeks post-infection (WPI). Secondary y-axis and dotted line (blue) shows water oxygen levels (% of air saturation). Statistical significance levels are presented in each plot after performing a Peto & Peto modification of the Gehan-Wilcoxon test between the curves for each group; Ctrl vs PRV (4, 7 and 10 WPI), Ctrl vs PRV-H (7 and 10 WPI) and PRV-H vs PRV (10 WPI). NS indicates not significant. (Paper I)*

An innate antiviral immune response is mounted against PRV in erythrocytes, peripheral blood, heart, spleen and head kidney as soon as the virus is detected in blood (16,17,45,46,356, Paper III). Virus production is an energy demanding process, and virus production occupies the cellular translational machinery. This could compromise the translation of proteins important for oxygen transport in the erythrocytes, like hemoglobin, and thereby reduce the hypoxia tolerance of the fish. At 10 WPI, the inflammatory changes in the heart reached peak levels and the PRV RNA level in blood and heart was reduced. The Hb levels in the PRV infected group were similar to the non-infected group at 10 WPI. This suggests that the reduced hypoxia tolerance in the PRV infected group at this time-point may be caused by the inflammation in

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the heart. Also in other experimental HSMI studies, the inflammation in the heart reached peak levels 8 – 10 weeks post PRV challenge (45,356). The inflammatory changes may impair the myocardial function and the cardiac pumping capacity, which is in line with the lower maximum heart rate detected in the PRV infected group at 10 WPI. Thus, cardiac inflammation may pose a possible explanation to why the PRV infected group showed a significantly lower hypoxia tolerance than the non-infected group at 10 WPI. The cardiac performance in the fish was investigated at 10 WPI by measuring the temperature-dependent maximum heart rate and is discussed in more detail in a later section.

Taken together, the PRV infected group have one factor in common at both 7 and 10 WPI; the inflammatory responses initiated by PRV. This may explain the reduced hypoxia tolerance observed in this group at both time-points. Furthermore, the low Hb level in the infected group at 7 WPI may also influence the hypoxia tolerance and needs further attention. Thus, information about the oxygen-carrying capacity, maximum heart rate and gene expression of inflammatory and erythropoiesis-related genes at 7 WPI could have explained the mechanisms in play. Moreover, a study on experimentally induced anemia in Atlantic salmon could elucidate the effects of Hb reduction on the hypoxia tolerance.

In studies investigating factors that affects the hypoxia tolerance in salmonids, genetic differences between families, differences in growth rate between strains and cardiac myoglobin levels have been found to play a role (154,156). In our trial, the experimental fish were from the same family and thus of the same genetic origin. However, measurements of cardiac myoglobin levels could have provided additional information to our study.

The improved hypoxia tolerance in the infected group observed at 4 WPI was unexpected (Figure 12 A). This may be due to either a higher body mass in the PRV infected group compared to the controls at this time-point, or due to host responses initiated by the early PRV replication in the erythrocytes. The latter needs to be elucidated in future studies. In a linear regression analysis, no effect of body mass on ILOS levels was detected in the compiled data or at the separate time-points (Paper I). This indicates that the increased hypoxia tolerance observed at 4 WPI may have been due to other factors. Nevertheless, several authors have suggested that hypoxia tolerance in fish is affected by body mass (120,126,154,157). Possible explanations to why larger individuals have a better hypoxia tolerance than smaller fish are a reduced metabolic rate and larger glycogen storages to be utilized in anaerobic glycolysis

during hypoxia (126). However, Roze and co-workers (2013) could not detect a firm link between body mass on hypoxia tolerance during a HCT performed on rainbow trout (154).
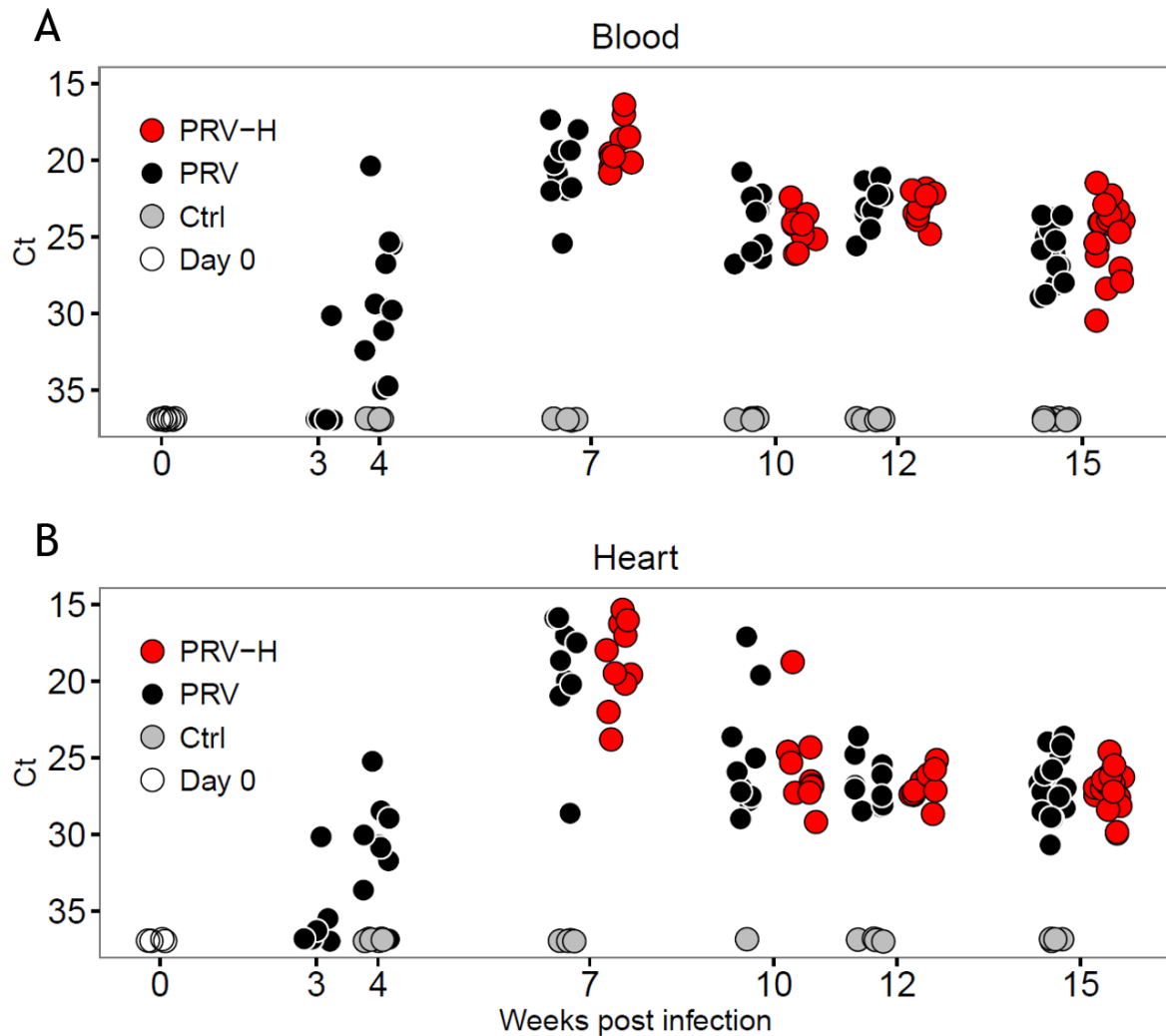
Figure 13. *PRV RNA Ct values in blood (A) and heart (B) (Paper I). Naïve fish sampled at Day 0 (white dots), non-infected controls (Ctrl, grey dots), PRV-infected (PRV, black dots) and PRV-infected fish exposed to periodic hypoxic stress (PRV-H, red dots), at each time-point during the infection trial. Weeks post-infection (WPI) are indicated on the x-axis. Ct value ≥ 37.0 indicates no virus RNA detected.*

5.2 Transient hypoxic episodes improve the hypoxia tolerance in PRV infected Atlantic salmon but did not affect HSMI development

Exposure of transient hypoxic episodes in one of the PRV-infected groups, i.e. the PRV-H group, at 4, 7 and 10 WPI did not have any detectable effects on the PRV levels in heart and

blood during the 15 week challenge trial (Figure 13). Nor did the disease development in the heart seem to be affected by transient hypoxic episodes (Paper I). There were no obvious differences in PRV RNA Ct values in blood and heart between the infected groups. Futhermore, no differences were detected in the histopathological scores except for a lower trend in the PRV-H group at 12 WPI (Paper I). This is in line with a study showing no effect on the development of pancreas disease during chronic hypoxia in Atlantic salmon (338). Furthermore, at 10 WPI the PRV-H group showed a significantly better hypoxia tolerance compared to the PRV infected group not exposed to transient hypoxia.

At 7 WPI, the PRV-H group had experienced only one previous hypoxic episode (at 4 WPI), which did not show any effect on the hypoxia tolerance (Figure 12B). However, a possible effect of the previous hypoxic episode may have been masked by the low Hb level in the PRV-H group at 7 WPI (Figure 14). At 10 WPI, the hypoxia tolerance was improved in the PRV-H group, which by this time-point had experienced two previous hypoxic episodes (at 4 and 7 WPI) (Figure 12C). Furthermore, the HCT performed at 10 WPI was 19 days after the last transient hypoxic episode experienced by the PRV-H group (at 7 WPI), suggesting a long lasting effect of the previous hypoxia exposure.

The pre-exposure of transient hypoxic episodes may have initiated protective myocardial mechanisms in the heart in the PRV-H group. Zebrafish myocytes have shown increased regenerative capacity after myocardial hypoxia or physical injury (420–422). The trend towards a lower histopathological score in the PRV-H group at 12 WPI may indicate an improved myocardial regeneration and less inflammation. Gene expression analysis to explore expression of myocardial injury markers and inflammatory responses could have provided some additional information.

Another explanation for the improved hypoxia tolerance in the PRV-H group at 10 WPI may be a preconditioning effect triggered by the previous hypoxic episodes. A preconditioning effect is a protective cardiac response initiated by previous stress stimuli such as hypoxia. Preconditioning effects in response to environmental hypoxia have been investigated in several fish species. *In situ* cardiac performance was not reduced following exposure to 15 minutes of anoxia in preconditioned rainbow trout hearts. The hearts received a preconditioning stimulus consisting of 5 minutes of anoxia and, after 20 minutes of normoxia, the hearts were exposed to 15 minutes of anoxia (423). A similar study was performed on hearts from hypoxia tolerant

rainbow trout and no preconditioning effect of 5 minutes of severe hypoxia was observed on the *in situ* cardiac performance following exposure of severe hypoxia (30 minutes) (424). Furthermore, the absence of a preconditioning effect in hypoxia tolerant rainbow trout was confirmed in a study performed by Overgaard and co-workers (147). These data suggests a clear variation in the cardiac response to a hypoxic stimulus between hypoxia sensitive and tolerant populations of rainbow trout. In zebrafish (*Danio rerio*) previously exposed to 48 hours of severe hypoxia, the survival time was improved during a second exposure to severe hypoxic conditions (425). Yang et al. (2013) detected an improved hypoxia tolerance in juvenile southern catfish (*Silurus meridionalis*) after being exposed to 15 days of cyclic hypoxia (10 hours of 3.0 mg/L DO and 14 hours of 7.0 mg/L DO), which also suggests a preconditioning effect (426).

The protective mechanisms initiated by the hypoxic preconditioning are not fully understood in fish. Possible explanations may be a physiological improvement of the myocardial contractility or improvement of the cellular ability to cope with oxidative damage. In hearts of the epaulette shark (*Hemiscyllium ocellatum*) preconditioned with hypoxia, an increased transcription of genes that improve oxygen and energy homeostasis was detected (427). This is interesting data, which needs to be confirmed in salmonids when investigating a possible priming effect of hypoxia in this species. Hypoxia-inducible factor 1α (HIF-1α) is shown to be increased in response to hypoxia and initiates a multitude of responses improving the hypoxia tolerance in fish (93,428). A higher and faster responsiveness of the upregulation of HIF-1α genes in individuals having experienced previous hypoxic episodes may be one mechanism explaining a preconditioning effect. Gene expression levels of HIF-1α before and immediately after the transient hypoxic episodes could be a marker for the differences in hypoxia tolerance between the infected groups in our studies. Acclimation to hypoxia was investigated in rainbow trout to elucidate if the increased Hb-O_2 affinity gained during chronic hypoxia could improve the physical performance of the fish. However, exposure to two weeks of hypoxia (40 mmHg PO_2) did not affect the performance during swimming respirometry (143).

Increased hemoglobin (Hb) levels in response to the transient hypoxic episodes could have improved the hypoxia tolerance observed in the PRV-H group at 10 WPI. However, the Hb concentration tended to be lower in the PRV-H group compared to the PRV-infected group at 10 WPI ($p = 0.07$) (Figure 14). This suggests that the Hb levels in the PRV-H group was not increased by the hypoxic episode 19 days earlier, and could not explain the improved hypoxia

tolerance compared to the PRV-infected group at 10 WPI. In rainbow trout, the Hb concentration started to increase after 24 hours of exposure to moderate hypoxia (55 % O_2 saturation) or 4 hours of severe hypoxia (30 % O_2) (135). The increase in Hb concentration stayed high for 9 and 6 days in the moderate and severe hypoxia groups, respectively. The release of erythrocytes into the circulation by splenic contraction and biosynthesis of Hb was suggested to cause the acute and long term increase in hemoglobin (135). However, it is not clear for how long the Hb levels are increased after the fish has returned to normoxic conditions.
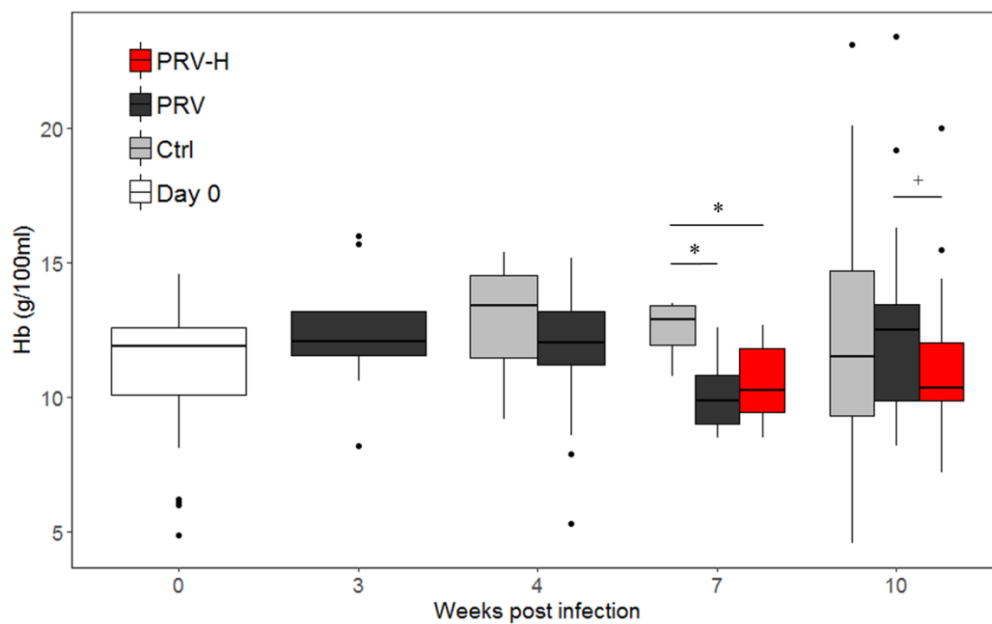
Figure 14. *Hemoglobin concentration in whole blood (Paper I). Hemoglobin (Hb) concentration (y-axis) in non-infected controls (Ctrl, grey), PRV-infected (PRV, black) and PRV-infected exposed to periodic hypoxic stress (PRV-H, red). The lower and upper border of boxes indicates the 25th and 75th percentiles, respectively and the centerline indicates the 50th percentile. The upper and lower whiskers correspond to the highest and lowest value of the 1.5*IQR (inter-quartile range). Significance is indicated by * with a p < 0.05 and + with a p = 0.07.*

5.3 Hemoglobin levels are reduced at peak PRV RNA levels in the heart

During the acute stages of PRV infection the erythrocytes are the main target cells (15,16). The circulating erythrocyte population of teleost fish consists of immature and mature cells. In rainbow trout blood, 81.9 %, 10.6 % and 7.5 % of the erythrocytes were categorized as mature, immature and post-mature, respectively (85). Furthermore, the young/immature salmonid erythrocytes are shown to have a higher total RNA content and a higher transcriptional and

translational activity than older erythrocytes (98). This could indicate that immature erythrocytes could be more efficient virus producers than mature cells. Hypoxia induces transcription of erythropoiesis-related genes in Atlantic salmon (92), which may indicate induced erythropoiesis and increased ratio of young erythrocytes relative to old/mature cells. Consequently, PRV-infection coinciding with an excess of young erythrocytes could possibly compromise the Hb synthesis. The changes in blood hemoglobin concentration during PRV infection in Atlantic salmon have not been reported until the study performed in Paper I. In this study, both PRV infected groups had significantly lower blood Hb concentration (g/100 ml whole blood) compared to the non-infected group at 7 WPI (Figure 14). At this time-point, the PRV RNA Ct values reached peak levels in the heart (Figure 13B). Pooled data from 7 and 10 WPI showed a significant positive correlation (r = 0.41, $p < 0.01$) between the Hb concentration and PRV RNA Ct values in the heart, suggesting a reduction in Hb concentration with increasing virus RNA levels, i.e. lower Ct levels, in the heart (Paper I). No correlation was detected between PRV RNA levels in blood and Hb concentration, despite the high levels of PRV RNA in blood at 7 WPI (Figure 13A). These results may suggest that the Hb concentration in blood is reduced by the PRV infection in Atlantic salmon. However, this need to be confirmed in future studies.

A recent study have shown that genes related to erythropoiesis are induced in head kidney and spleen following a PRV infection in post-smolts (356). Measurements of hematocrit (HTC) in experimentally induced HSMI have not indicated that PRV infection causes anemia in Atlantic salmon. However, in other fish species, i.e. Coho salmon and rainbow trout, infection with other PRV strains are associated with anemia (40,429). Hence, PRV induced anemia in Atlantic salmon may occur under certain conditions.

5.4   PRV infection reduces cardiac performance

HSMI is characterized by severe epicarditis and panmyocarditis with infiltration of mononuclear cells (53), which primarily consists of CD8$^+$ lymphocytes (45). In the challenge trial described in Paper I, the histopathological changes in heart reached peak levels at 10 WPI. A significantly lower maximum heart rate (f_{Hmax}) at 19 °C was detected in the PRV infected post-smolts 10 weeks post infection (Paper I). The optimum temperature (T_{opt}) for aerobic scope was lower in the PRV infected group, which may suggest that infection and/or inflammation impair the cardiac capacity.

Although cardiac inflammation is evident in other important viral diseases in farmed Atlantic salmon like PD and CMS (55), there are no reports on the effects these virus infections may have on cardiac function in salmonids. Experimentally PMCV infected Atlantic salmon post-smolts have been studied with respect to heart function, showing significantly reduced heart rate and lower f_{Hmax} 9 weeks post PMCV challenge (pers. comm. Jørgensen S.M. and Timmerhaus G.). However, moderate but increasing levels of PRV RNA were also detected in the hearts of these fish, which may have influenced the results. Nevertheless, these data suggest an impaired cardiac function due to PMCV infection in Atlantic salmon (pers. comm. Jørgensen S.M. and Timmerhaus G.). Hearts from ISAV infected Atlantic salmon have also shown reduced cardiac performance (159). This effect was linked to the infection of endothelial cells in the blood vessels and endocardium and the subsequently diminished nitric oxide signaling pathway (159). In addition, parasitic infections causing heart lesions in rainbow trout and Atlantic cod have been shown to reduce the cardiac performance in the diseased fish (430,431).

The PRV infected group in our study that had experienced two previous hypoxic episodes (at 4 and 7 WPI), had a similar f_{Hmax} as the non-infected groups, which is in line with the hypoxia tolerance observed in the HCT at 10 WPI. The higher maximum heart rate in the PRV-H group compared to the PRV infected group may reflect an improved cardiac function due to the previous hypoxic episodes. Such effects have been shown in sea bass exposed to hypoxia (392). In that study, improved myocardial contraction *in vitro* during hypoxia was demonstrated in tissue isolated from hypoxia-tolerant individuals, which suggested that the hypoxia tolerance of the heart may reflect the hypoxia tolerance at organism level (392).

5.5 Hemoglobin oxygen affinity is reduced in PRV-infected fish exposed to hypoxic episodes

The blood of the PRV-H group had a lower hemoglobin-oxygen affinity 10 weeks post infection, i.e. 19 days since the last hypoxic episode, compared to the control group, which was detected by an increased P_{50} in the PRV-H group and a right shift in the hemoglobin-oxygen dissociation curve (ODC) (Figure 15) (Paper I). A decreased Hb-O_2 affinity can improve the oxygen unloading from the erythrocytes in the respiring tissues (103). In contrast, the expected immediate response to hypoxia according to previous studies is an increased Hb-O_2 affinity, which ensure an increased oxygen loading in the gills (75,81). The different fish species and

time from the hypoxia exposure, may explain the different effects on Hb-O_2 affinity between our Paper I and Weber et al (1979) (81).
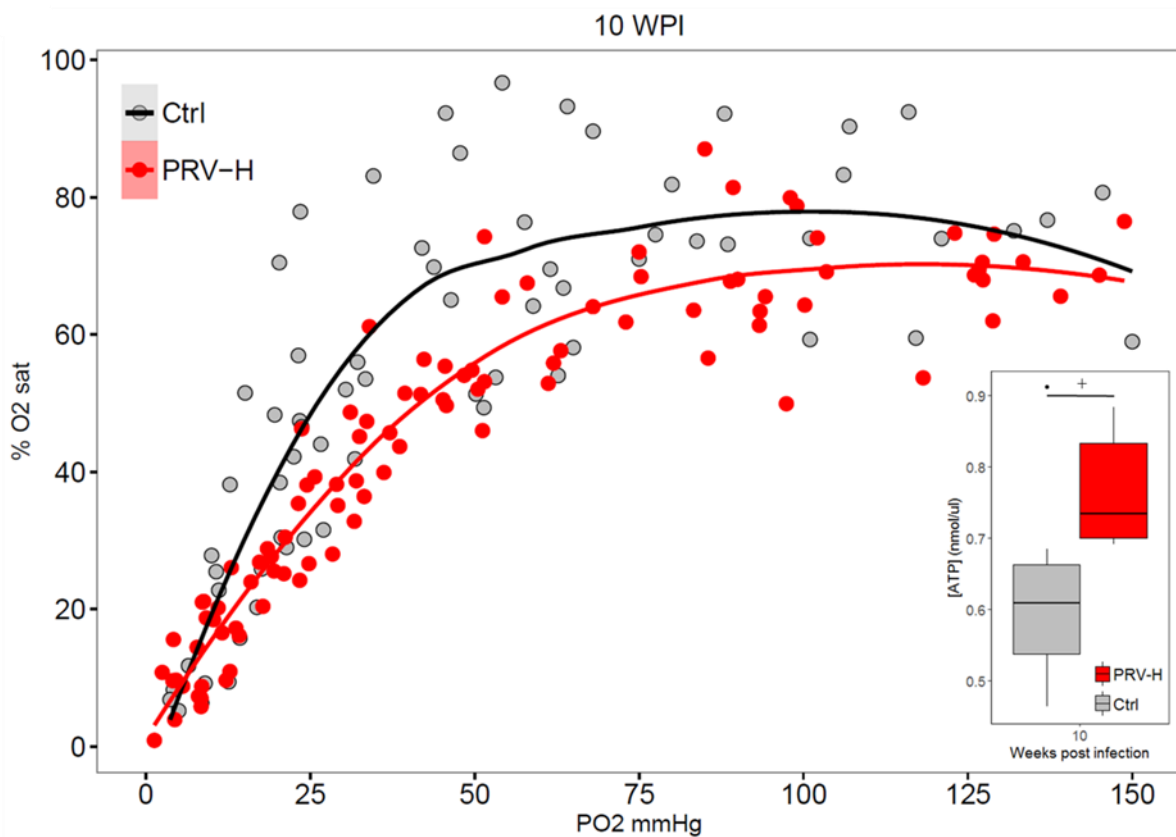
Figure 15. *Blood oxygen binding affinity in PRV-infected fish exposed to periodic hypoxic stress (PRV-H, red curve) and non-infected controls (Ctrl, black curve) at 10 weeks post-infection (WPI). Hb-O_2 dissociation curves (ODC) relating partial pressure of oxygen (P_{O2}; x-axis) with Hb-oxygen saturation (y-axis). ATP concentrations are shown in inset with significance level (p = 0.07, indicated by +).*

The ATP concentration tended to be higher in the PRV-H blood compared to the control group ($p = 0.07$) in our study (Figure 15, inset), and may explain the right shift in the ODC. This is in contrast to reduced erythrocytic ATP/GTP levels after hypoxia in other studies on salmonids (74,81,136). The intra-erythrocytic organic phosphates, i.e. ATP and GTP, are important regulators of the oxygen affinity to hemoglobin in fish (75,103). A reduction in ATP concentration is a common acute response to hypoxia in teleost fish, which results in an increase of the hemoglobin-oxygen affinity (42,81,134,432). Furthermore, the hypoxia-induced reduction in ATP levels in rainbow trout was normalized within four days after being returned to normoxic water (432). This suggests that the effects on the hemoglobin-oxygen affinity due to the hypoxia-induced reduction in ATP concentrations are limited in time. When the ODC were measured in our study (i.e. 10 weeks post infection) it was 19 days since the last transient

hypoxic episode of the PRV-H group. Hence, a possible reduction in the ATP concentration during the hypoxic episode at 7 WPI would be expected to normalize within the measurement of the Hb-O$_2$ affinity at 10 WPI. It is still unclear if the mechanisms behind the increased ATP level in the PRV-H group could be due to the PRV infection in the erythrocytes or the exposure to two transient hypoxic episodes.

To the author's knowledge, this is the first report regarding effects of virus infection in salmonids on blood ATP concentration. Malaria (*Plasmodium berghei*) is a well-known mammalian erythrocytic parasite. In malaria-infected mice erythrocytes, the Hb concentration and intracellular pH was reduced in addition to a reduced Hb-O$_2$ affinity demonstrated by a right shift of the oxygen hemoglobin dissociation curve (433). In that study, the ATP production of the parasite explained the reduced Hb-O$_2$ affinity in the infected erythrocytes (433). Future experimental PRV challenge trials should investigate the ATP content in the erythrocytes to study the development during the infection. The possible pre-conditioning effect on the erythrocytic ATP level should also be investigated.

Furthermore, future studies should include ODC measurements at earlier stages of the PRV infection to be able to elucidate the effects of an on-going virus replication within the erythrocytes. Performing ODC measurements immediately after ending the hypoxic episodes of longer duration (days) may further provide information on effects of PRV infection in erythrocytes.

5.6 Experimental PRV infection protects against pancreas disease

In the challenge trial of Paper II, PRV infected fish were co-infected by introducing SAV shedders 4 and 10 weeks post PRV challenge (Figure 16). Both SAV subtypes common in Norwegian aquaculture, i.e. SAV3 and SAV2, were included in the experiment. The SAV subtypes are found in geographically separated areas in Norway, however, with some overlap (434). SAV3 is reported to cause higher mortality than SAV2 (360). Therefore, the challenge trial aimed to investigate possible differences between these subtypes in a co-infection with PRV. However, no significant differences in virus RNA levels and histopathological changes were detected after infection with the two subtypes in our study, and hence, the SAV infection is generally addressed without differing between the subtypes.
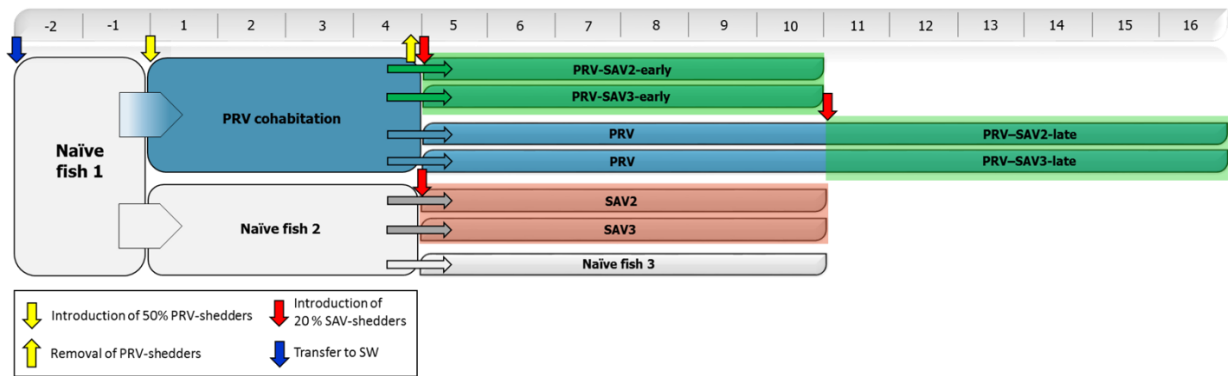
Figure 16. *The PRV-SAV co-infection challenge trial. The timeline above indicates weeks post PRV shedder introduction (WPC-PRV).* ↓ *indicates transfer to sea water (SW).* ↓ *and* ↑ *indicates introduction and removal, respectively, of PRV shedder fish.* ↓ *indicates introduction of SAV shedders. "Naïve fish 1" indicate the experimental fish before virus challenge, "Naïve fish 2" indicate fish dedicated to be SAV shedders or SAV controls at 4 WPC-PRV and "Naïve fish 3" indicate fish dedicated to be SAV shedders at 10 WPC-PRV. Blue box named "PRV cohabitation" indicates cohabitant fish exposed to 50% PRV shedders from 0 – 4 WPC-PRV. Blue box named "PRV" indicates PRV cohabitants from 4 to 10 WPC-PRV without PRV shedders. Boxes indicating a 6 week period of exposure to 20% SAV shedders are colored red (SAV only) and green (PRV-SAV). Co-infection induced at 4 WPC-PRV and 10 WPC-PRV is denoted PRV-SAV-early and PRV-SAV-late, respectively. (Paper II).*

In this experiment, PRV-SAV co-infected fish showed significantly reduced development of pancreas disease compared to fish infected with SAV alone. The protection was observed both when SAV was introduced early after PRV challenge, i.e. 4 WPC, and late, i.e. 10 WPC. PD related histopathological changes in pancreas and myocardium, i.e. important target organs for SAV, were significantly lower in the co-infected groups compared to the SAV controls (Figure 17). Furthermore, the mean condition factor (k-factor) of the co-infected groups was higher than the SAV controls. A reduced k-factor is a common finding during PD and is caused by reduced or absent appetite in the diseased fish (360,435). Taken together, our results suggest that the PRV infection reduces the inflammation triggered by SAV infection. However, neither PRV RNA levels nor HSMI development was affected by the introduction of SAV (Paper II).
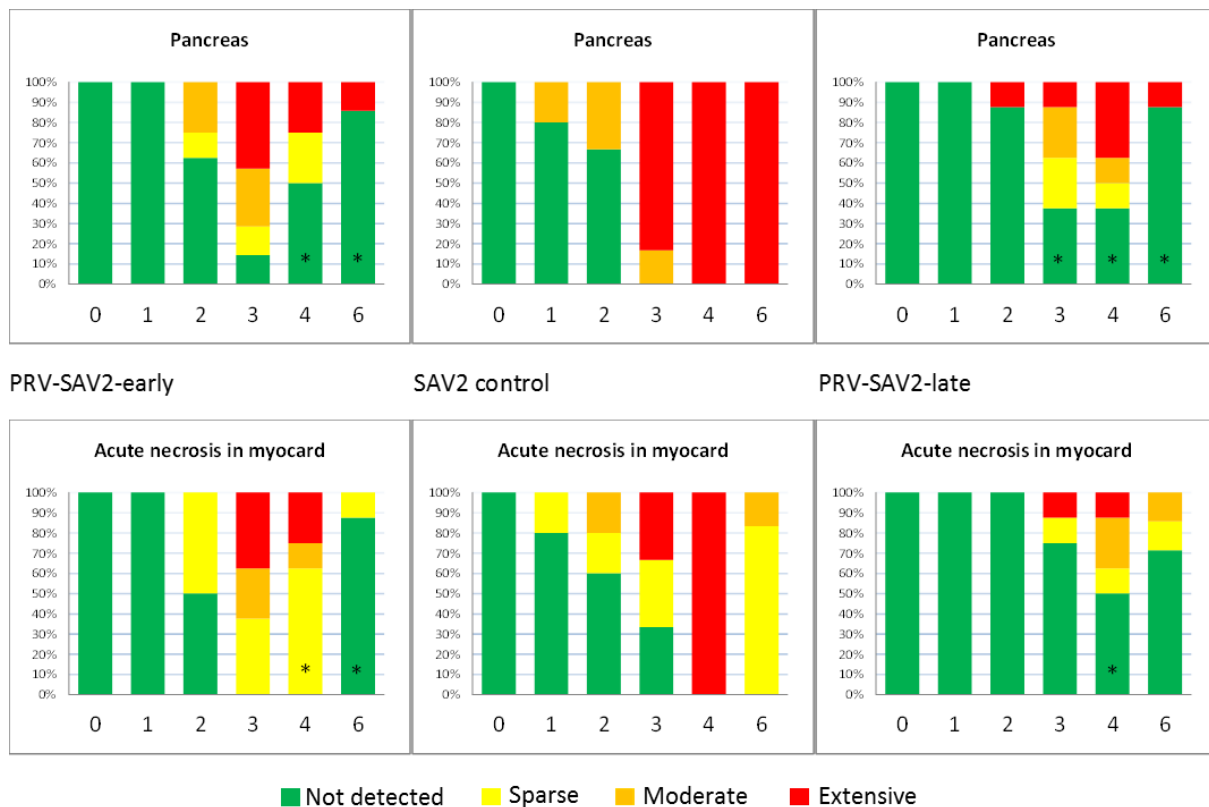
Figure 17. *Histopathological evaluation of pancreas and acute necrosis in myocard in SAV infected and co-infected groups. PRV-SAV2-early indicates introduction of SAV2 shedders 4 weeks post PRV challenge. PRV-SAV2-late indicates introduction of SAV2 shedders 10 weeks post PRV challenge. X-axis denotes weeks after introduction of SAV-shedders. Significance is determinet by p < 0.05 and is indicated by *.*

In the PRV-SAV co-infected fish, the atrophy of the exocrine pancreas was significantly lower compared to the SAV controls (Paper II). Pancreatic tissue is not an affected organ during HSMI (55), which suggests that the observed protection may be due to the systemic immune response mounted during the primary PRV infection. Acute myocardial necrosis is considered a consistent finding during early PD development (391) and was scored histopathologically to assess the effect of the co-infection in the heart. The early and late co-infected groups had significantly lower scores of acute myocardial necrosis when compared to the SAV controls. Interestingly, at 4 and 6 weeks post SAV shedder introduction, the late co-infected groups presented a trend of less severe scores of acute myocardial necrosis compared to the early co-infected groups (Figure 17). Normally, peak inflammatory changes in the heart during HSMI occurs 10 weeks post PRV challenge (356, Paper I) and this may have affected the interpretation of the PD-related myocardial necrosis score. Another explanation is that the immune responses triggered by the PRV infection protects better against SAV-mediated tissue injury in this phase.

After 10 weeks post PRV challenge, the inflammatory changes in the HSMI heart is gradually resolving (Paper I).

An oligonucleotide microarray was performed in order to study transcriptomic responses during the co-infection (Paper II). We analyzed gene expression in hearts collected from co-infected and SAV controls 4 and 6 weeks after the SAV challenge. Increased expression of specific genes related to myocardial changes during SAV infection have been described earlier (357). An up-regulation of these genes was detected in the hearts in our study, and the level of gene expression was reduced in the co-infected groups when compared to the SAV controls (Paper II), in line with the lower histopathological scores in the co-infected groups. Specifically, Ct values of neuropeptide Y-1 was significantly correlated ($r = 0.70$) with scores of myocardial degeneration and inflammation (Paper II). Neuropeptide Y has several effects on the inflammatory responses and cardiomyopathy in mammals (436,437) and may serve as a marker gene for evaluating the severity of myocardial injury in SAV infected Atlantic salmon.

5.7   Interaction between co-infecting viruses

The outcome of a co-infection is dependent on the type of pathogens infecting the host, virulence of the pathogens and the timing of the introduction of the secondary pathogen (438). Infection with viruses activates the innate antiviral immune system. Hence, at the time of the secondary infection, the activated innate antiviral response of the host is likely to act against the subsequent challenge with another virus.

The PRV-infected groups reached peak levels of PRV in blood and heart at 5 and 6 WPC, respectively. The PRV levels then gradually decreased until 10 WPC from which they remained stable until the end of the experiment, i.e. 16 WPC (Paper II). During the early co-infection, SAV RNA was first detected in the co-infected blood and hearts two weeks post SAV shedder introduction and reached peak levels after three weeks post SAV challenge. The SAV RNA levels in the co-infected groups were significantly lower at 3, 4, and 6 (in blood) and at 4 and 6 (in heart) weeks post SAV challenge compared to the SAV controls. In the late co-infected groups, SAV RNA was first detected two and three weeks post SAV3 or SAV2 challenge, respectively, and peaked four weeks post SAV challenge in both co-infected groups (Paper II). The SAV RNA levels in the co-infected groups showed a larger variation than in the SAV control group, which may indicate a reduced SAV infection efficiency in the PRV-infected

individuals. Futhermore, both PRV and SAV antigens were detected by immunohistochemistry in heart sections, which confirmed the presence of both viruses in some individuals (Paper II).

Hedrick and co-workers (1994) performed a co-infection challenge trial in which rainbow trout fingerlings (body mass: 1.3 – 2.6 g) were infected by the non-virulent Cutthroat trout virus (CTV) and subsequently challenged with IHNV (439). The results showed significantly reduced mortality in the co-infected groups (439). In another experimental co-infection, protection was observed towards a secondary IHNV infection in rainbow trout juveniles (body mass: 5 - 9 g) already infected by the non-virulent Chum salmon reovirus (CSRV) (440). The surviving co-infected fish had significantly lower IHNV neutralization titers compared to the IHNV infected controls (440).

Johansen et al (2014) performed an IPNV bath challenge of Atlantic salmon parr, which were subsequently i.p. infected with PRV at seawater transfer, i.e. 12 weeks after IPNV challenge, (441). The researchers did not detect any effect on PRV RNA levels or HSMI development, 7 and 10 weeks post PRV infection (441). Hence, the antiviral immune response initiated during a chronic IPNV infection did not affect the subsequent PRV infection. However, as IPNV and PRV are both non-enveloped dsRNA viruses and expected to initiate a similar immune response, an earlier introduction of PRV to the IPNV infected fish could potentially have initiated a protective effect. In IPNV-ISAV co-infected Atlantic salmon, reduced mortality was observed when ISAV was introduced to IPNV infected fish three weeks after IPNV challenge. In the same study, increased mortality was observed when IPNV infected fish were challenged by a secondary infection of *Vibrio salmonicida* (442). The authors suggested that there was a reduced production of reactive oxygen species in head kidney macrophages due to the acute IPNV infection and hence a reduced antibacterial response (442).

The duration of the cross-protective effects demonstrated in Paper II lasted for at least 10 weeks post PRV challenge. In other experimental co-infections where a protective effect have been observed, the lag between the primary and secondary infections varies between 2 – 8 weeks (439,440,442,443). This may be due to differences in the ability of the primary infecting virus to stimulate a long-lasting innate immune response. The timing and administration route of the subsequent infection and the life stage of the host is also likely to be of importance. A recent study suggests that the susceptibility to SAV in Atlantic salmon post-smolts is affected by the time after seawater transfer (361). The authors detected a lower degree of SAV infection in fish

exposed to SAV nine weeks post seawater transfer compared to fish challenged by SAV two weeks post seawater transfer. A compromised immune response during smoltification may explain the differences in susceptibility to SAV (361). Johansen and co-workers (2016) detected a reduced expression of 300 immune genes during smoltification and one week post seawater transfer in Atlantic salmon (444). However, the magnitude and duration of the immunosuppressive effect after seawater transfer is not clear. Additional stressors at this stage in the Atlantic salmon production cycle, like the transportation from the freshwater facility to the marine production site and mixing of different smolt groups could also suppress immunity (335,445).

In our trial, there were no transportation or mixing of groups. The PRV infected groups were challenged by SAV 6 and 12 weeks post seawater transfer in the early and late co-infection, respectively. Therefore, the differences in the outcome of the early and late co-infection could have been influenced by the differences in time since seawater transfer. Moreover, age and size of the fish at the time of infection have shown to affect the susceptibility to viral infections in salmonids (446,447). Thus, these factors may also have influenced the SAV infection kinetics in the early and late co-infected groups.

An additional finding was that the SAV shedders added to the PRV infected fish were infected by PRV after 6 weeks of cohabitation (Paper II). During the early co-infection, the SAV2 shedders presented slightly higher PRV RNA levels in the heart compared to the SAV3 shedders. Furthermore, the SAV3 RNA levels in the co-infected fish showed a stronger correlation to myocardial necrosis than SAV2 RNA levels. Taken together, these observations could reflect a difference in virulence and antiviral response between these SAV subtypes in Atlantic salmon (360).

5.8   Immunological mechanisms of the PRV-SAV protection

The gene expression of a set of antiviral immune genes was analyzed by RT-qPCR in order to study immunological mechanisms contributing to the PRV-mediated protection against SAV infection. (Paper III). Several interferon-stimulated genes (ISGs) were chosen based on their reported actions against the alphavirus replication cycle. Blood, heart and spleen from the PRV infected fish at the time of SAV shedder introduction was analyzed. The ISGs were significantly up-regulated 4 and 10 weeks post PRV challenge in all three tissues (Paper III). This confirms

a long lasting antiviral state triggered during PRV-infection, which could explain the observed protection in the co-infected groups. These results support other studies showing a strong antiviral innate immune response during PRV infection (17,45,356,357). The erythrocytes are important target cells for PRV and several ISGs were up-regulated in the PRV infected blood cells (Figure 18). The immune response mounted in blood cells may play an important role in the systemic protection from the secondary SAV infection, illustrated by the protective effect on the exocrine pancreas tissue in the co-infected groups. The genes of the antimicrobial peptides β-defensin and hepcidin were differentially expressed in blood, heart and spleen (Paper III). However, both β-defensin and hepcidin were significantly up-regulated in blood 4 weeks post PRV challenge, and suggest an antiviral response that was diminished later in the PRV-infection.
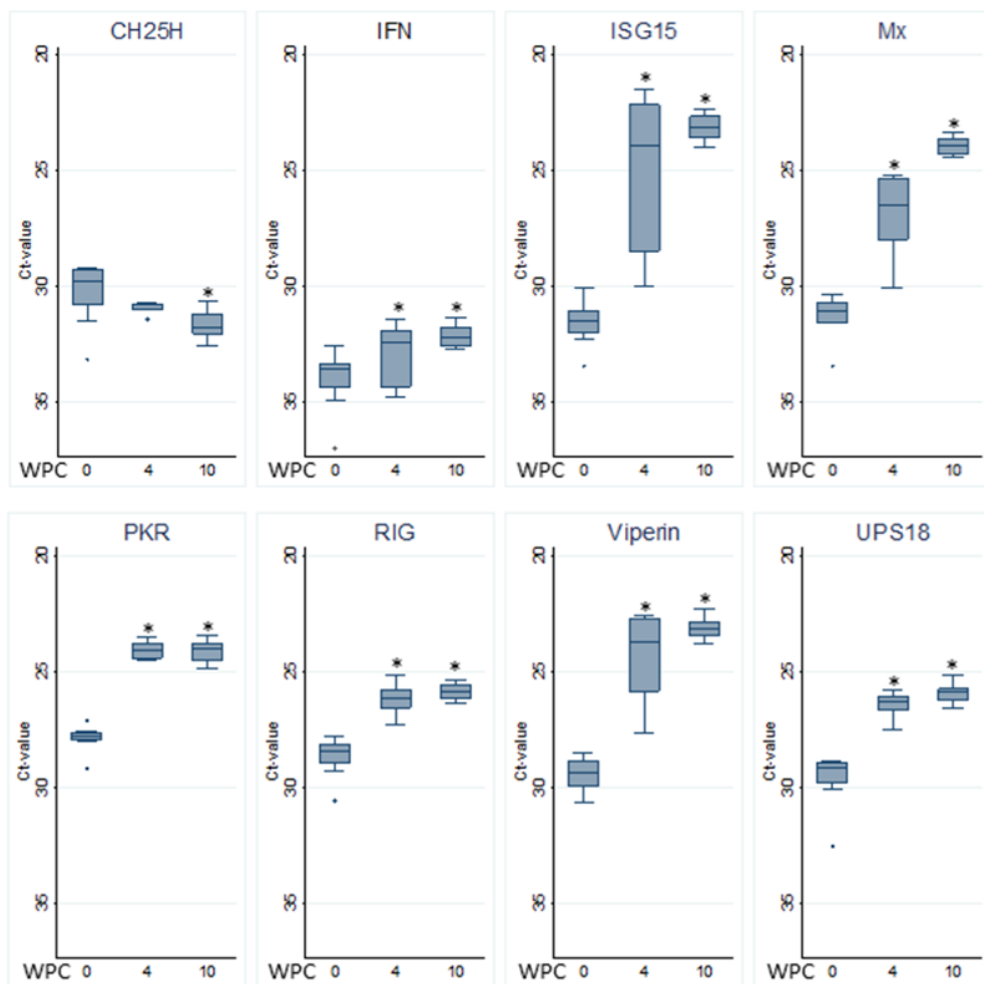
Figure 18. *Gene expression (Ct value) of immune genes in blood in PRV infected fish according to weeks post PRV challenge (WPC). The middle bars (lines within each box) represent the median, box region are limited by 25th percentile and 75th percentile, and whiskers marks the upper and lower adjacent value (1.5*inter-quartile range). Outliers are marked with dots. * indicates significant difference from 0 WPC (p<0.05) (Paper III).*

In a recent co-infection trial performed in Sockeye salmon with a North American isolate of PRV, i.p. infected fish were challenged by IHNV immersion 14 days after PRV infection (358). Both viruses were detected in the co-infected fish, but no effects of PRV infection on IHNV superinfection or typical histopathological changes were detected (358). Furthermore, the long-lasting innate antiviral immune response initiated in Atlantic salmon observed in Paper III contrasts the minor innate antiviral immune response mounted by PRV infected Sockeye salmon (358). The fact that PRV infected Sockeye salmon does not mount a strong innate antiviral immune response may explain the lack of any protective effect on the subsequent IHNV infection.

The Atlantic salmon belongs to the genus *Salmo*, while the Sockeye salmon belongs to the evolutionary distant genus *Oncorhynchus*. During the evolution, these two genera split apart approximately 15-20 million years ago (448), and the large evolutionary distance indicates that pathogenesis of a PRV infection in one species does not mirror the pathogenesis in the other species. Taken together, this suggests a difference in host susceptibility between Atlantic and Sockeye salmon or a virulence difference of the different PRV subtypes in Norway and North America. In a similar experimental PRV-IHNV co-infection in Atlantic salmon, PRV strongly protected against IHNV infection and a significantly reduced mortality was observed in the co-infected group (pers. comm. Vendramin N., 2017).

To explore potential cross-protective effects mediated by the humoral immune system, a SAV neutralization test was performed on plasma from PRV, SAV and PRV-SAV infected fish (Paper III). Cross-neutralizing effects were observed in plasma from some PRV-infected individuals, but they were lost after heat-treatment, indicating that they were complement-dependent. In contrast, the neutralizing effects of plasma from SAV-infected individuals on SAV itself were sustained after heat-treatment, indicating the expected presence of SAV specific antibodies (208). The co-infected fish had a lower SAV neutralizing antibody titer six weeks post SAV challenge compared to the SAV controls, in line with a lower level of infection. Similarly, in a CSV-IHNV co-infection study in rainbow trout, the serum neutralizing titers were reduced in the co-infected fish when compared to IHNV infected controls. The authors suggested that a cell-mediated immune response also contributed to protection during the co-infection in addition to the innate antiviral mechanisms (440). In contrast, increased neutralizing titers were observed in CTV-IHNV co-infected rainbow trout (439), which suggest differences in the immune response initiated by the initial virus infection.

The complement system in teleost fish inactivates pathogens in serum or mucus when activated by binding to the pathogen or to antibody-antigen complexes (205,415,449,450). Hence, in a neutralization test, the complement proteins needs to be inactivated if the aim is to determine the titer of specific antibody mediated neutralization (207,208). Unlike the antibody response against IHNV, which is complement dependent to gain fully neutralizing activity (207), neutralizing SAV antibodies are proposed to be complement independent (208). The plasma used in the neutralizing assay in Paper III was heat-treated to inactivate complement. The treatment did not affect the neutralizing properties of plasma from the SAV controls, indicating that complement-independent antibodies mediated the neutralization. In the co-infected groups, the plasma neutralizing activity was lower than in the SAV controls, indicating a lower titer of SAV specific antibodies. This may reflect a reduced B cell mediated response due to a lower level of SAV antigens.

Interestingly, the untreated plasma from four PRV infected fish (N = 16) had neutralizing effects on SAV four weeks post PRV challenge. No neutralizing effect was detected at 7 and 10 weeks post PRV challenge. After heat-treatment of the plasma, one sample from a PRV infected individual still neutralized SAV. This may indicate that PRV infection up-regulates heat-sensitive humoral factors (possibly complement) with non-specific neutralizing properties toward SAV *in vitro*. Moreover, the sustained neutralizing effect in one of the PRV-infected samples after heat-treatment is interesting.

5.9 PRV and SAV RNA levels in field samples from co-infected farmed Atlantic salmon

In the Longitudinal study in Paper III, a PRV-SAV co-infection was investigated in randomly selected apparently healthy fish during a field outbreak of PD in two net pens at one seawater site. The fish were PRV-positive before SAV was detected, and the study could be considered as a secondary challenge with SAV. A negative correlation (r = -0.22, $p < 0.05$) between PRV and SAV RNA levels was detected in co-infected individuals (N = 159), indicating that higher PRV-levels led to reduced infection with SAV (Figure 19 A) (Paper III). This may be due to an activated immune response in the PRV-infected fish. However, a similar trend but no significant correlations were detected when analyzing the separate time-points in the Longitudinal study (Figure 19 B). These time-point variations could be caused by individual variation in the development of the infection.
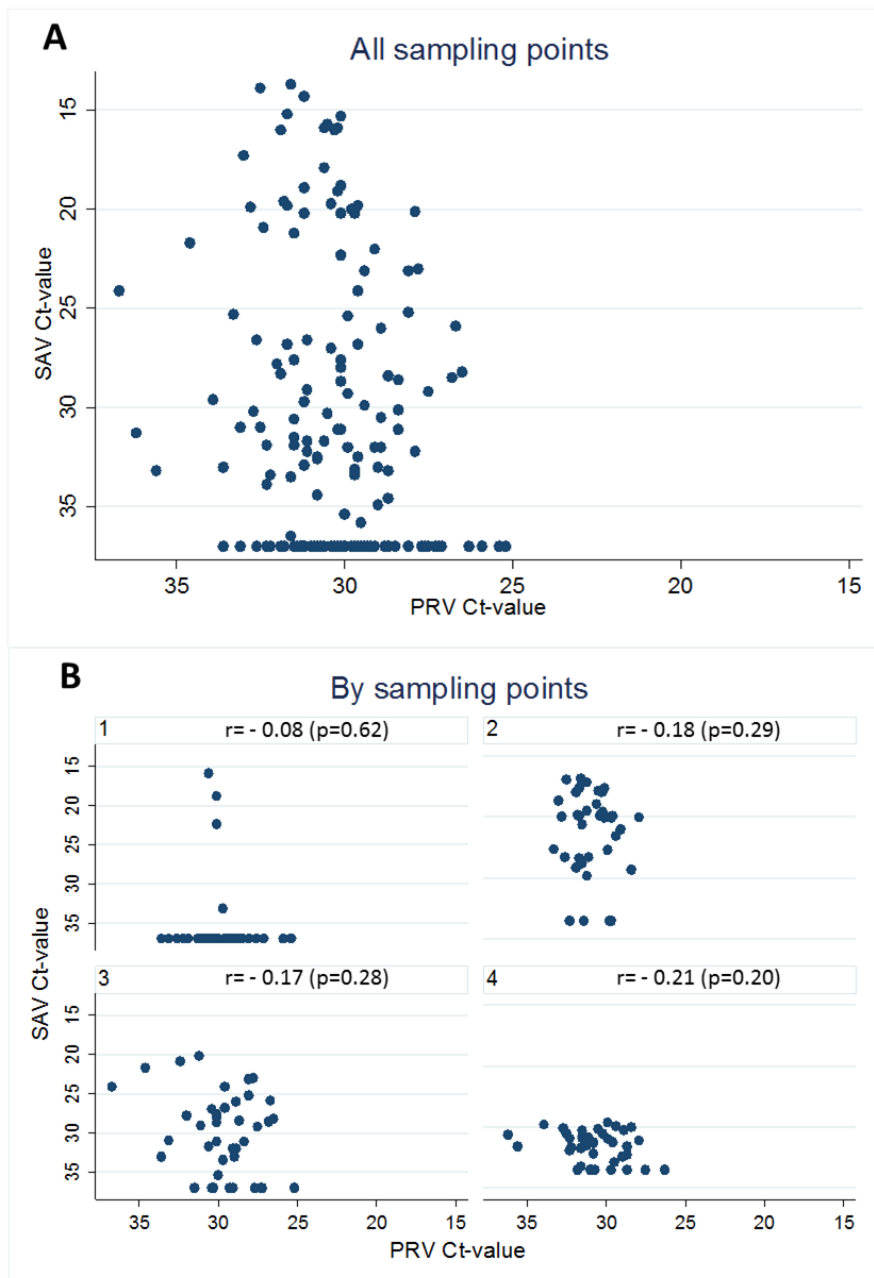
Figure 19. *PRV and SAV Ct values in fish sampled in the Longitudinal study. A. Plot of the compiled data points. Spearman rank correlation coefficient of – 0.22 (p < 0.05). B. Plot of data at each time-points including the Spearman rank correlation coefficients with respective p values. (Paper III).*

The results in the Longitudinal study was in line with the experimental PRV-SAV co-infection. However, the time between the two infections was larger in the Longitudinal study as the fish population at the production site was first diagnosed with HSMI 5 months ahead of the detection of SAV in the study pens. This indicates a long lasting PRV infection within the source population. The time lag between the primary PRV infection, the duration and magnitude of the antiviral immune response is expected to affect the outcome of the secondary infection.

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In the Screening data, samples from newly dead or moribund fish (N = 307) showed a positive correlation (r = 0.23, $p < 0.05$) between PRV and SAV RNA levels (Paper III). This indicated that PRV and SAV RNA levels increased in concert. In moribund fish, the immune response may be diminished, and a higher susceptibility to a secondary infection could be expected in severely immunocompromised individuals.

The discrepancy between the correlations detected in the Longitudinal study and the Screening data may reflect that PRV positive moribund fish are more prone to SAV infection compared to apparently healthy PRV positive individuals. Wiik-Nilsen and co-workers (2016) investigated a range of field cohorts diagnosed with myocarditis (25). In one cohort, a negative correlation between PMCV and SAV RNA Ct values was detected in co-infected individuals (N = 13), however no correlation was detected between PRV and SAV RNA Ct values (25). Although these results indicate a possible interplay between co-infecting viruses in farmed Atlantic salmon, more epidemiological and experimental studies are needed before drawing firm conclusions.

The samples in both the Longitudinal study and the Screening data were collected in the endemic SAV2 geographical area, which is supposed to be devoid of SAV3. SAV2 was recently introduced to this area in Norway, where SAV had not previously been detected (451). The SAV2 subtype induce similar PD specific histopathological changes as SAV3, however the mortality is lower in SAV2 infected fish (360). The fact that the fish in both field data series were infected by SAV2 may have led to a different result than if the fish had been infected with SAV3.

5.10 Implications for the aquaculture industry

As shown in Paper I, PRV infected fish are less tolerant to hypoxia. These findings suggest that PRV infected farmed Atlantic salmon may be more sensitive to hypoxic stress, which is important information in a field situation. Furthermore, a tendency of lower T_{opt} in PRV infected fish detected in Paper I indicates that PRV diseased hearts are less tolerant to stress at higher temperatures than non-infected fish. This aspect should be investigated further. Salmonids have a relatively low optimum temperature for maximum heart function (452) and because the handling of PRV infected salmon at late summer, i.e. peaking of seawater temperatures, could be detrimental due to the reduced amount of dissolved oxygen. The increase in drug resistance

among sea lice populations have increased the use of mechanical de-louse treatments (6). Thus, to avoid handling of the PRV-infected fish at risk temperatures and critical oxygen levels, an alternative strategy should be discussed during the peak infection period. This could reduce losses and improve fish welfare due to excessive handling.

Interestingly, the improved hypoxia tolerance observed in the infected group when previously exposed to two transient non-lethal hypoxic episodes, suggests a pre-conditioning effect. Our results are in line with other studies which observe a protective effect of cardiorespiratory conditioning before introducing a stressor (400,427). Vindas and co-workers (2016) exposed pre-smolts to chronic physical stress, and observed a lower responsiveness to acute stress in the hypothalamus and brain stem and an increased growth-rate for up to 10 weeks post stress (453). Nevertheless, it remains to be determined if Atlantic salmon pre- or post-smolts could be pre-conditioned to better resist acute or chronic stress and infections after seawater transfer.

We showed that primary PRV infected Atlantic salmon mounted an innate antiviral immune response that may have restricted a secondary SAV infection for at least 10 weeks in an experimental co-infection. This suggests a beneficial effect of a PRV infection when SAV is introduced within a certain period after PRV challenge. However, in a net pen at sea, containing up to 200 000 individuals, the infection dynamics will differ from an experimental study tank. For instance, the timing and dose of infection, and environmental factors like temperature and oxygen saturation will vary and may influence the interference between primary and secondary infections. Nevertheless, our findings are a relevant contribution to the discussion on which factors affects the outcome of PD outbreaks regarding mortality and reduced growth.

The field data sets in Paper III represent the first documentation of virus levels in a PRV-SAV co-infection before and during a PD outbreak in farmed Atlantic salmon. These data may suggest that PRV infected fish have a lower probability of being co-infected by SAV and that dead or moribund co-infected fish show the contrary, possibly due to differences in immunological status. Our experimental data supports the field observations. Further investigations are needed to obtain more insight of the complexity of co-infections under field conditions, and especially regarding the influence from environmental factors.

6   Main conclusions

The challenge studies presented in this thesis have demonstrated that PRV infection has other consequences in Atlantic salmon besides causing HSMI.

- PRV infection reduces tolerance for hypoxia in early and late stages of the infection
- PRV infection reduces erythrocyte hemoglobin level and the hemoglobin-oxygen binding affinity
- HSMI diseased hearts have a lower maximum heart rate and a lower optimal temperature for maximum cardiac performance
- Previous exposure of PRV-infected fish to hypoxic episodes has a protective effect on acute hypoxia tolerance
- PRV infected fish are protected from a secondary SAV infection and PD development for at least 10 weeks after PRV infection
- Innate immune responses are activated for 10 weeks, and may play a role in the protection
- Field data consisting of PRV-SAV co-infected Atlantic salmon revealed a negative correlation between PRV and SAV RNA levels in apparently healthy fish and a positive correlation in dead and moribund individuals

7 Future perspectives

The aim to increase the Atlantic salmon production in Norway fivefold by 2050 requires control of both biological and technical challenges during the freshwater and marine grow-out phase of the production cycle. To obtain this expansion of the aquaculture, and to obtain societal acceptance of the production, the industry must control infections, minimize escapees, and collect and facilitate reuse of waste products, all to minimize the environmental footprint and secure good fish welfare.

PRV is ubiquitous in seawater reared farmed Atlantic salmon and PRV variants cause problems in several other salmonid fish species as well (31,40,429). PRV are possibly also associated with melanised spots in Atlantic salmon fillets, which causes economically significant quality reduction (454). Futhermore, PRV reduce the hypoxia tolerance of Atlantic salmon (Paper I). Preventive measures need to be taken to reduce the infection pressure of PRV on farmed salmonids. This should be based on increased knowledge on the virus, pathogenesis of the infection, modes of spread, impact on wild salmonids, and should aim to reduce the economic and eventual environmental impact of the infection.

The past years, PRV and HSMI outbreaks have been reported increasingly often also in freshwater facilities in Norway (6). Challenges with PRV infection in pre-smolts are likely to increase in the future, both by causing HSMI outbreaks and by affecting the hypoxia tolerance and cardiac performance of the fish. Furthermore, the possible influence of PRV on smoltification, i.e. the transitional phase preparing the freshwater parr to a seawater environment, is important to elucidate. In a national survey performed in 2013 by the Norwegian food safety authority, the responders related a high share of the mortality during the first three months after seawater transfer to reduced smolt-quality (5). Thus, it is important to investigate the consequence of a PRV infection prior to smoltification to clarify if the infection leads to losses after seawater transfer.

PRV is a non-enveloped virus, which is known to survive long outside the host and to be resilient toward disinfection. The future use of closed containment production facilities, either on land or at sea, may therefore increase the problems with resident virus strains, which may result in a higher infection pressure of single or multiple infectious agents. This needs to be addressed when large-scale production is initiated in such facilities. Furthermore, in the closed containment facilities, the water is recycled and strict control of water temperature and O_2/CO_2

content within the tank is crucial. If these environmental parameters reach critical tolerance levels of the PRV infected fish, this may lead to physiological imbalance and/or aggravated disease development.

In Paper I, we detected a reduced hypoxia tolerance and a lower maximum heart rate at 19 °C in PRV infected fish. These findings should be pursued due to possible complications for a future salmonid aquaculture affected by global warming. The subsequent effects of impaired cardiac performance in PRV infected farmed salmon during sea lice treatments during periods of high water temperatures should be investigated. Thus, the hypoxia and thermal tolerance of PRV infected salmon reared at 14 – 18 °C would add important information about disease development, cardiorespiratory consequences and how farmed salmon react to these environmental challenges.

The results in Paper II showed a protective effect of a primary PRV infection during a secondary SAV infection. The PRV-SAV co-infection should be studied further to investigate the duration of the innate immune response initiated by PRV. More knowledge is needed regarding the outcome of the co-infection if SAV is introduced earlier or later and how the co-infection affects the growth rate. Furthermore, the possible difference in outcome between a PRV-SAV2 and PRV-SAV3 co-infection should be addressed further. In Paper II, we detected a trend of lower impact of PRV on a SAV2 co-infection. Follow-up studies should emphasis differences in the immune response initiated by the two SAV subtypes. The impact of stress during a PRV-SAV co-infection should also be investigated as handling and hypoxic stress is common in a field setting and may affect the outcome of the co-infection.

A PRV infection can persist for at least 12 months after infection in salmonids (48). It will be necessary to characterize the immune response towards PRV more thoroughly to better understand why PRV RNA levels can persist in fish long after the inflammation have resolved. How does the low-grade replication in the persistent phase hide from the immune system? Is it necessary for PRV to hide from the immune system at all? Does the low-grade replication take place in other cell types than the erythrocytes? Leukocytes have been shown to harbor IPNV and IHNV infection in surviving individuals and hence, asymptomatic carriers of these virus infections are common (455,456). This may explain the detection of PRV RNA by RTqPCR long after the acute phase of replication has ended. The potential effect on a low-grade persistent PRV infection on the cardiorespiratory capacity or other physiological effects should be

addressed. Furthermore, the possibility of reactivating a low-grade PRV infection due to a stress stimuli or a co-infection is necessary to know due to the ubiquitous presence of PRV in the marine farmed salmon.

The different outcomes from PRV infections in farmed salmonids, point out the need for elucidating potential virulence differences between the PRV strains. The reported accumulated mortality for HSMI varies from $0 - 20\ \%$, a variability which should be elucidated. Differences in the outcomes of PRV infections in Atlantic, Sockeye and Coho salmon and rainbow trout, could also indicate host differences in the immune reactions toward PRV. These studies could contribute to the understanding of immunological mechanisms and thus point out relevant targets for vaccine development. Vaccines plays a pivotal role in the preventive strategy against several pathogens. However, whole virus vaccine production needs a sustainable and efficient virus cultivation system, i.e. cell cultures. A continuous cell-line to propagate PRV *in vitro* would therefore be beneficial. This would also be a valuable tool in the further research on PRV to better understand the pathogenesis of HSMI.

The parallel use of field data, screening data, diagnostic data and experimental data is important in the future handling of current and emerging pathogens in fish farming. An integrated use of both field and experimental data, allows the researcher to understand the complex nature of the current diseases in today's aquaculture. Furthermore, this strategy will be beneficial in the handling of future diseases. The use of field data in statistical modeling is increasing in the aquaculture. These models are used to predict the spread of salmon lice (457) and in control strategies of virus induced diseases (458–460). The use of statistical modeling on the occurrence and severity of PRV infections in relation to production parameters may detect subtle interactions that affects an HSMI outbreak and may be used to initiate preventive measures. Furthermore, these models can be used to validate experimental data to field settings, which would ease the extrapolation of experimental data into applicable knowledge in the aquaculture. To achieve this, the aquaculture industry and academic institutions needs to cooperate and systematically collect and store both field and research data for epidemiological use.

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9 Scientific papers I - III

Paper I

Hypoxia tolerance and responses to hypoxic stress during heart and skeletal muscle inflammation in Atlantic salmon (*Salmo salar*)

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Abstract

Heart and skeletal muscle inflammation (HSMI) is associated with *Piscine orthoreovirus*

(PRV) infection and is an important disease in Atlantic salmon (*Salmo salar)* aquaculture.

Since PRV infects erythrocytes and farmed salmon frequently experience environmental

hypoxia, the current study examined mutual effects of PRV infection and hypoxia on

pathogenesis and fish performance. Furthermore, effects of HSMI on hypoxia tolerance,

cardiorespiratory performance and blood oxygen transport were studied. A cohabitation trial

including PRV-infected post-smolts exposed to periodic hypoxic stress (4 h of 40 % O_2; PRV-

H) at 4, 7 and 10 weeks post-infection (WPI) and infected fish reared under normoxic conditions (PRV) was conducted. Periodic hypoxic stress did not influence infection levels or histopathological changes in the heart. Individual incipient lethal oxygen saturation (ILOS) was examined using a standardized hypoxia challenge test (HCT). Prior to hypoxic stress (4 WPI), infected fish were more hypoxia tolerant than non-infected fish. At 7 WPI, i.e. peak level of infection, both PRV and PRV-H groups exhibited reduced hypoxia tolerance compared to non-infected fish. Three weeks later (10 WPI), during peak levels of pathological changes, reduced hypoxia tolerance was still observed for the PRV group while PRV-H performed equal to non-infected fish, implying a positive effect of the repeated hypoxic stress treatments. This was in line with maximum heart rate (f_{Hmax}) measurements, showing equal performance of PRV-H and non-infected groups, but lower f_{Hmax} above 19 °C as well as lower temperature optimum (T_{opt}) for aerobic scope for PRV, suggesting reduced cardiac performance and thermal tolerance. In contrast, the PRV-H group had reduced hemoglobin-oxygen affinity compared to non-infected fish. In conclusion, Atlantic salmon suffering from HSMI have reduced hypoxia tolerance and cardiac performance, which can be improved by preconditioning fish to transient hypoxic stress episodes.

Introduction

Heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon (*Salmo salar*) is an important viral disease in Norwegian aquaculture [1,2]. HSMI has also been reported in farmed Atlantic salmon in Scotland [3] and Chile [4]. The disease is characterized by panmyocarditis (including cellular epicarditis), myocyte necrosis and inflammation in red skeletal muscle [2]. *Piscine orthoreovirus* (PRV) is associated with HSMI [5] and has been detected in farmed salmonids in Ireland, Chile, Japan, BC Canada and Canada/US Pacific Coast [6–12] and in wild

47 salmonids [13]. PRV is non-enveloped and have a segmented double-stranded RNA genome,

48 and belongs to the genus *Orthoreovirus* in the family *Reoviridae* [5,14].

49 Erythrocytes are important target cells for PRV and virus replication in these cells has been

50 confirmed both *in vivo* and *in vitro* [15,16]. Similar to higher vertebrates, piscine erythrocytes

51 secure transport and exchange of oxygen (O_2) to metabolic active tissues. In contrast to

52 mammals, fish erythrocytes are nucleated with functional organelles like ribosomes and

53 mitochondria, giving them additional properties such as protein synthesis, metabolic activity

54 and immunological functions [17–21]. During PRV infection, high levels of viral transcripts

55 and proteins are detected in the erythrocytes, inducing transcription of genes involved in innate

56 immune responses [16,22]. Hence, one may hypothesize that the viral processing and immune

57 responses may affect hemoglobin production and oxygen binding and transport capacity of

58 infected erythrocytes. This may have consequences for the hypoxia- and stress-tolerance of

59 infected fish. Furthermore, the extensive epicarditis and inflammation of the myocardium

60 associated with HSMI may have additional negative consequences for cardiorespiratory

61 functions and blood transport. Apart from some studies showing reduced cardiac performance

62 to infectious cardiomyopathies in fish [23–25], more knowledge is needed with regards to the

63 secondary cardiorespiratory effects of viral infections, and in particular PRV infection which

64 targets both erythrocytes and cardiac muscle in salmonids.

65 During their lifespan of 2-3 years, farmed Atlantic salmon encounter several stressful

66 challenges. Intensive rearing conditions (i.e. high stocking density and growth rate) combined

67 with hypoxic episodes, crowding stress and reduced physical condition can be detrimental

68 during viral infections. Naturally occurring acute and chronic hypoxic episodes, ranging from

69 30-70 % oxygen saturation, are described during the seawater production phase [26–28]. Such

70 episodes can be caused by a combination of natural fluctuation in oxygen levels (i.e. alternating

71 water currents, tidal exchange, temperature, net fouling and algae blooms) and variations in

72 oxygen consumption by the fish themselves (i.e. biomass, stress, crowding and behavior)

73 [27,28]. Water temperature and salinity are inversely correlated to the solubility of oxygen and

74 hence affects the consequences of hypoxia in fish. In a cyclic hypoxia trial, Atlantic salmon

75 post-smolts showed reduced appetite and growth when exposed to oxygen saturation lower than

76 70 % at 16 °C [29]. Hypoxia during infections represents an additional stressor that may

77 compromise energy resources needed for the immune response. Such mechanisms are poorly

78 studied and need attention as hypoxia clearly affects important physiological functions in fish.

79 There is only one published study on viral infection during hypoxia in Atlantic salmon, showing

80 no effect on pancreas disease (PD) development during chronic hypoxia (60-65 % O_2

81 saturation, 12 °C) [30].

82 In teleosts, two important physiological responses to hypoxia are increased cardiac output

83 [31,32] and induced erythropoiesis [33]. The most common viral diseases in seawater-farmed

84 Atlantic salmon in Norway, i.e. HSMI, PD and cardiomyopathy syndrome (CMS), all induce

85 characteristic pathological lesions in cardiac muscle [34]. Such lesions may affect

86 cardiomyocyte contractility and cardiac function, and hence lead to impaired cardiac output

87 themselves, which may be further compromised in response to additional stressors such as

88 environmental hypoxia. Induced erythropoiesis in response to hypoxic stress leads to increasing

89 numbers of circulating erythrocytes. In turn, this may increase the potential target cells for PRV

90 and could therefore affect the susceptibility and development of HSMI. Overall consequences

91 may be exacerbated disease development, increased morbidity and mortality, reduced animal

92 welfare and increased economic losses. This illustrates the importance of clarifying whether

93 PRV and associated cardiac pathology sensitizes the fish to environmental hypoxia or general

94 crowding stress. The aims of this study was to examine whether periodic transient hypoxic

95 stress affects virus levels and disease development during PRV infection, and whether HSMI,

96   alone or in combination with periodic hypoxic stress, affects hypoxia tolerance,

97   cardiorespiratory performance and blood oxygen transport in Atlantic salmon.

98

Materials and Methods

Experimental fish and rearing conditions

101   Seawater adapted Atlantic salmon from the SalmoBreed strain (Bergen, Norway) were used in

102   the study, including N=705 fish implanted with passive integrated transponder (PIT; Jojo

103   Automasjon AS, Sola, Norway) tags and N=705 unmarked fish. Tagged fish were i.p. injected

104   with PIT tags two weeks before transfer to the research facility (VESO Vikan, Namsos,

105   Norway). After transfer, fish were acclimatized to brackish water (25‰ salinity) for two weeks

106   before PRV infection. Prior to infection, fish were confirmed negative for PRV, Infectious

107   pancreatic necrosis virus (IPNV) and Salmon pancreas disease virus (SPDV) using quantitative

108   reverse transcription PCR (RT-qPCR). During the infection trial, fish were kept in filtered and

109   UV-radiated brackish water (25‰ salinity), 12 °C (±1 °C) and continuous light. Fish were fed

110   a standard diet using a ratio of 1.5 % of total biomass per day, and were starved for 24 h prior

111   to handling and sampling. Before sampling, fish were euthanized by bath immersion containing

112   benzocaine chloride (200 mg/L water) (Apotekproduksjon AS, Oslo, Norway) for 5 min. The

113   challenge trial was approved by the Norwegian Animal Research Authority and performed in

114   accordance with the recommendations of the current animal welfare regulations: FOR-1996-

115   01-15-23 (Norway).

Experimental infection trial

117   The inoculum was pelleted blood cells collected from a previous cohabitation trial, which was

118   the first passage in experimental fish originating from a field outbreak of HSMI in 2012. The

119   preparation of the inoculum is described earlier [35], and was confirmed negative for IPNV,

Infectious salmon anemia virus (ISAV), SPDV, Piscine myocarditis virus (PMCV) and Atlantic

salmon calicivirus (ASCV) using RT-qPCR [35]. At Day 0, shedder fish (N=470) were i.p.

injected with 0.1 ml of the inoculum, marked by removal of the adipose fin and distributed

equally into two fiber glass tanks (infected groups), each containing non-infected PIT tagged

fish (N=235 per tank). A third fiberglass tank (control group) contained equal numbers of non-

infected fish tagged by adipose fin removal (N=235) and PIT (N=235).

The infection trial lasted for 15 weeks. Time-points for tests and samplings are displayed in

Table 1. Experimental groups are denoted Ctrl (non-infected controls), PRV (infected) and

PRV-H (infected and exposed to periodic hypoxic stress, as described in detail below).

Sampling for disease development (PRV RNA and histopathological evaluation) was done from

all groups before any handling of fish was initiated at 4, 7 and 10 weeks post-infection (WPI).

Maximum heart rate and hemoglobin-oxygen affinity measurements were conducted at 10 WPI,

when peak levels of pathology were expected. Acute hypoxia challenge test was performed at

4, 7 and 10 WPI. Details on the methods used and sampling procedure performed after each

test are described below.

WPI	0	4			7			10			12			15		
	Day 0	PRV-H	PRV	Ctrl	PRV-H	PRV	Ctrl	PRV-H	PRV	Ctrl	PRV-H	PRV	Ctrl	PRV-H	PRV	Ctrl
Sampling for disease developement	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X
Hypoxia challenge test			X	X	X	X	X	X	X	X						
Heart rate measurement								X	X	X						
Hemoglobin oxygen dissociation curve								X		X						
Periodic hypoxic treatment		X			X			X								

Table 1. Experimental groups and sampling time-points.

Overview of experimental groups included in the infection trial and weeks post-infection (WPI)

at which the specific groups were sampled or tested. "Sampling for disease development" was

139   performed before any test were performed at 4, 7 and 10 WPI. For the other test categories, fish

140   were sampled directly after finishing the respective tests. PRV-H: PRV-infected fish exposed

141   to periodic hypoxic stress, PRV: PRV-infected fish, Ctrl: non-infected fish.

142

143   Mortalities during infection were negligible. One fish (0.6 %) died in the PRV-H group and two

144   fish (1.2 %) died in each of the PRV and control groups.

145   **Sampling procedures**

146   Organ samples were collected throughout the infection trial from all groups on which

147   physiological tests were performed (Table 1). Weight and length was registered and Fulton's

148   condition factor (k-factor) was calculated (k-factor = weight in grams/(length in cm)^3 * 100).

149   Twelve fish were sampled prior to PRV cohabitation as negative controls. Heart and gill

150   samples for histopathological evaluation were collected and fixed in 10 % phosphate buffered

151   formalin. Within 48 hours after sampling, the formalin was replaced with 70 % ethanol and

152   stored at 4 °C until further handling.

153   Two pieces of heart tissue (~2 mm^3) for RT-qPCR analysis were collected on 1.0 ml tubes

154   (FluidX Ltd., UK) prefilled with 0.5 ml RNA*later™* (Ambion Inc., USA). Blood was collected

155   from the caudal vein using lithium heparin-coated vacutainers (Vacutest klima, Italy) with 20

156   G Venoject needles. Immediately after sampling, a sub-sample consisting of 20 µl heparinized

157   blood from each individual was added to 1.5 ml Eppendorf tubes prefilled with 1.0 ml Drabkin's

158   solution (Sigma-Aldrich, USA) for hemoglobin (Hb) analysis. Unless stated otherwise, all

159   samples were stored chilled (5-7 °C) and dark until shipment to the laboratory. Tissue samples

160   on RNA*later™* were placed chilled overnight and at -20 °C until analyzed.

161   **RNA isolation and RT-qPCR**

162 From the heparinized blood sample 300 µl was removed and shipped chilled (5-7 °C), together

163 with the heart samples on RNA*later*™, to PatoGen Analyse (Ålesund, Norway) for RT-qPCR

164 analysis. PatoGen Analyse performed RNA extraction and RTqPCR analysis for PRV RNA in

165 heart and heparinized blood. The RT-qPCR assay, validated according to the ISO17025

166 standard, target PRV transcripts and is described elsewhere [36]. Elongation factor 1α (EF1α)

167 served as an internal reference gene [37] for all RT-qPCR assays performed. Samples having a

168 PRV Ct lower than 37.0 were defined as positive.

169 **Histopathological analysis**

170 Samples for histopathology were processed and stained with hematoxylin and eosin following

171 standard procedures. The sections from heart and gill were scored blindly and a subsample of

172 hearts (32 % of samples scored) was re-scored blindly by a second examinator.

173 Histopathological changes in heart related to HSMI (i.e. epicarditis and myocarditis in the

174 compact and spongy layers) were scored on a continuous visual analogue scale (ranging from

175 0-3) modified from Mikalsen *et al*. [38]. S1 Table displays the scoring criteria used.

176 **Periodic hypoxic stress**

177 At 4, 7 and 10 WPI the PRV-H group was treated with hypoxic stress by reducing oxygen

178 saturation in the tank to 40 % O_2 (±4 %) for four hours. The oxygen saturation of 40 % was

179 reached within 30 min after gradually reducing the water flow, and kept stable by adjusting the

180 water flow into the tank. The O_2 level was monitored by two oxygen probes (Handy Polaris,

181 OxyGuard, Denmark) placed 10 cm above the tank floor, one over the tank outlet and one in a

182 sector aside of the outlet. The fish was continuously monitored during the test and the level of

183 oxygen was recorded manually every 15 min. Sampling of 10 cohabitants was performed

184 immediately after ending the test and oxygen level was gradually normalized within one hour

185 by increasing the water inlet. The biomass in the tank was 39, 37 and 39 kg/m^3 at 4, 7 and 10

186   WPI, respectively, during the hypoxic treatments. Water temperature was kept at 12 °C during

187   the test. No mortality occurred during or after the test.

Acute hypoxia challenge test

189   Acute hypoxia challenge tests (HCT) were performed in accordance to previous studies [39,40]

190   at 4, 7 and 10 WPI. A water recirculation system with a closed, upright gas equilibrium column

191   was mounted in a separate 1.0 m fiberglass tank containing 425 l of brackish water. Thirty fish

192   from each group were transferred to the HCT tank and allowed to acclimate overnight before

193   the test. Tank biomass was 20.0, 25.7 and 37.2 kg/m^3 at 4, 7 and 10 WPI, respectively. The

194   HCT was initiated the next morning, consisting of a rapid decrease in oxygen saturation,

195   reaching approximately 25 % saturation within one hour, followed by a slower descent of

196   approximately 3-4 % saturation per hour until termination of the test. During the test, tank water

197   was pumped (Compact 600, EHEIM, Germany) into the gas equilibrium column containing bio

198   filters, where oxygen was exchanged with nitrogen (N_2) gas bubbled into the bottom of the

199   column. This ensured a homogenous mixing of N_2 and water, which was pumped back into the

200   tank and enabled control of ambient oxygen. The flow of N_2 was controlled by regulating using

201   a controller and solenoid valve (Yara Praxair AS, Oslo, Norway). Oxygen saturation and

202   temperature was measured and logged continuously from two OXROB10 optical oxygen

203   probes in the tank connected to a FireStingO2 oxygen meter (PyroScience GmbH, Aachen,

204   Germany). Oxygen probes were calibrated before each test. When the fish lost the ability to

205   maintain equilibrium (i.e. incipient lethal oxygen saturation (ILOS) was reached), they were

206   quickly removed from the tank and euthanized by a blow to the head. Corresponding time and

207   oxygen level was recorded along with identification of PIT tags to locate group affiliation. The

208   HCT was terminated when all fish had reached ILOS. Samples of heart in RNA*later*™ and

209   formalin and heparinized blood were collected from N=10 (4 WPI) and N=20 (7 and 10 WPI)

210   fish per group. As soon as practically possible after sampling, a sub-sample of 20 µl heparinized

211   blood from each individual was added to 1.5 ml Eppendorf tubes prefilled with 1.0 ml Drabkin's

212   solution for Hb analysis.

213 **Maximum heart rate measurement**

214   Temperature-dependent maximum heart rate (f_{Hmax}) was measured at 10 WPI on a subset of fish

215   (N=16 per group with mean body mass of 170 g) as described by Anttila *et al.* [41]. Briefly, an

216   electrocardiogram (ECG) was recorded with a chromel-A measuring electrode positioned

217   lightly on the skin just below the heart and a reference electrode positioned caudal to the heart,

218   in a small chamber receiving temperature-controlled aerated water (25 ‰ salinity, 12 °C) from

219   a Julabo circulating chiller/heater (F32 ME, Julabo GmbH, Seelbach, Germany) under mild

220   anesthesia (60 ppm MS-222, buffered to pH 7.0). The ECG signal was amplified (1000×, Grass

221   P55 amplifier, Astro-Med, Brossard, QC, Canada) and filtered (50 Hz line filter; low-pass: 30

222   Hz; high-pass: 0.3 kHz) before being stored in a PowerLab data acquisition system

223   (PL3508, PowerLab 8/35, AD Instruments Ltd, Oxford, UK). Heart rate was allowed to

224   stabilize for 30 min at 12 °C before an intraperitoneal injection of atropine sulphate (2.4 mg kg^{-1}

225   dissolved in 0.9 % NaCl; Sigma-Aldrich, Oslo, Norway) that blocked vagal inhibition of the

226   heartbeat. Water temperature was increased in 1 °C increments for a cumulative warming rate

227   of 10 °C h^{-1}, beginning 15 min after the atropine injection. At each temperature increment, both

228   the water temperature and f_{Hmax} were stable. f_{Hmax} was recorded at each temperature increment

229   by counting R-R intervals for final 15 heartbeats before another temperature increment. The

230   incremental heating was terminated at 20 °C, before cardiac arrhythmias were expected. After

231   finishing the test, the fish were euthanized routinely and samples were collected from the heart

232   in RNA*later*™ and formalin as well as heparinized blood from all tested individuals.

233 **Hemoglobin-oxygen dissociation measurement**

234   Hemoglobin-oxygen dissociation measurements were analyzed according to the Tucker method

235   [42]. Measurements were performed on blood sampled from the PRV-H (N=12) and control

236   group (N=12) at 10 WPI. Fish were euthanized routinely as described above and heparinized

237   blood (1.5 - 2.0 ml) was drawn from the caudal vein. Individual samples measuring less than

238   2.0 ml were pooled with the sample from a second individual within the same group. A total

239   amount of 2.0 - 2.5 ml blood was transferred into a rotating blood tonometer (handmade in

240   glass) and gassed with air or N_2 gas. The blood was incubated with 1.0×10^{-5} M propranolol

241   (Actavis, Iceland) in phosphate buffered saline to block the effects of adrenaline and nor-

242   adrenaline on the β-adrenoreceptors, affecting oxygen binding affinity of salmonid hemoglobin

243   [43]. Within the tonometer, the sample was kept at a temperature of 11-13 °C by circulating

244   chilled water around the tonometer. Successive desaturation of the blood was undertaken by

245   gassing the blood sample with nitrogen. At successive levels of oxygenation determined by

246   direct measurement of blood PO_2, the oxygen content of the blood was determined using the

247   Tucker method in a thermostatted TC500 Tucker Cell (Strathkelvin Instruments, Scotland). The

248   procedure was carried out on each blood sample to generate data from at least 5-7 different

249   levels of oxygenation. The PO_2 levels were coupled with the spectrophotometric measured

250   hemoglobin concentration of the sample. After finishing the measurements of the individual

251   sample, 20 µl of the heparinized blood was added to 1.5 ml Eppendorf tubes prefilled with 1.0

252   ml Drabkin's solution for Hb measurement, as described below.

253   **Hemoglobin measurement**

254   Hemoglobin concentration (g/100 ml blood) was measured spectrophotometrically as

255   cyanmethemoglobin on heparinized blood samples mixed 1:500 with Drabkin's solution by

256   determining the absorbance at 540 nm. The procedure was performed on a TECAN Sunrise

257   microplate reader (TECAN, Switzerland) according to the manufacturer's procedure for

258   Drabkin's reagent (Cat.no. D5941, Sigma-Aldrich, Scotland). The Hb concentration was

259   determined from a standard curve prepared from bovine Hb powder (H2500-1G, Sigma-

260   Aldrich, Scotland).

ATP measurement

262   Adenosine triphosphate (ATP) concentration (nmol/µl blood) was measured on heparinized

263   blood samples (100 µl) snap frozen on liquid nitrogen. The ATP concentration was calculated

264   by fluorometric detection of ATP using a colorimetric/fluorometric assay kit (Cat.no. MAK190,

265   Sigma-Aldrich, Scotland) on an EnSpire 2300 Multilable plate reader (PerkinElmer, USA)

266   according to the manufacturer's protocol. The amount of ATP in each well was obtained from

267   the standard curve and the ATP concentration in each sample was calculated as described by

268   the manufacturer.

Data analysis and statistics

270   The statistical analyses and plotting were performed in R (version 3.3.1) for all data.

271   Differences in viral RNA levels, ILOS, histopathological changes in heart, hemoglobin and

272   ATP levels between the groups were examined using the non-parametric Mann-Whitney

273   unpaired rank test. An unpaired Student's t-test was used to examine differences in k-factor and

274   weight. A linear regression analysis and Pearson correlation analysis (R; stats and corrplot

275   packages) was performed on individual weight and oxygen saturation levels from data collected

276   during the HCT's (both compiled and separately). Kaplan-Meier plots were generated from the

277   HCT by using the additional "survival" package in R. Differences between the curves were

278   tested by performing a Peto & Peto modification of the Gehan-Wilcoxon test embedded in the

279   same package. The data from the temperature dependent heart rate measurement was computed

280   by using the following additional packages: RColorBrewer, devtools, AquaR (github

281   "gtimmerhaus/aquaR")).

282   An oxygen dissociation curve was fitted by a local polynomial regression fit ("loess" function

283   in the "stats" package) through O_2 saturation and PO_2 registrations. Whole blood oxygen

284   content was calculated according to Tucker [40]. From these measurements, data was corrected

285   for Hb concentration after subtracting the physically dissolved O_2 according to [44] and the

286   percent saturation of Hb calculated. Data was then log transformed ($\log10((O_2/gHb)/(1-$

287     $(O_2/gHb))$ and $\log10\ PO_2$) according to [45] and a linear regression line was fitted through the

288   linearized data. The K_d (zero intercept) and Hill coefficient (n_H) was determined using

289   SigmaPlot 10.0 (Systat Software Inc, London, UK). The inverse log of the K_d is equivalent to

290   the P_{50}. A p-value < 0.05 was considered statistically significant for all data analyzed.

291   A Pearson correlation and linear regression analysis were performed (R; stats and corrplot

292   packages) for associations between PRV Ct values in heart and blood and Hb concentration.

293 # Results

294   **Body metrics**

295   From the start until termination of the infection trial (15 WPI), body weight increased (from

296   77.6 ± 1.7 g to 260 ± 3.4 g, N=33 and N=130) and condition factor remained unchanged (from

297   1.27 ± 0.014 to 1.23 ± 0.008, N=33 and N=130) for all groups (S2 and S3 Figs). There were no

298   significant differences in body weight or condition factor between infected groups (PRV versus

299   PRV-H group). However, compared to the Ctrl group, PRV-H had significantly lower body

300   weight ($p < 0.05$) and condition factor ($p < 0.01$) at 12 WPI, and the PRV group had

301   significantly lower weight ($p < 0.05$) at 10 WPI and condition factor at 10 ($p < 0.05$) and 12

302   WPI ($p < 0.01$) (S2 and S3 Figs). Body weight of PRV and PRV-H fish included in the HCT

303   was significantly higher compared to the Ctrl group at 4 WPI but not at the later time-points

304   (S4A Fig). Regression and Pearson correlation analyses of data recorded from all HCTs or at

305 each time-point separately, showed no effects of individual body weight on oxygen saturation

306 at ILOS ($r^2 < 0.03$) (S4B-D Figs).

Periodic hypoxic stress does not affect PRV RNA levels or HSMI development

308 In order to evaluate the effects of three short-term hypoxic stress treatments (40 % O_2 for 4 h)

309 on HSMI pathogenesis, virus RNA levels and histopathological changes were evaluated in

310 PRV-H and PRV groups at 4, 7, 10, 12 and 15 WPI (Figs 1 and 2). Non-infected controls were

311 negative for virus and pathological changes at all time-points. There were no differences in

312 virus RNA levels and histopathology scores between groups prior to the first hypoxia treatment

313 (4 WPI). After hypoxic stress, there were no significant differences in virus RNA levels in

314 blood and heart between the PRV-H and PRV groups at any time-point (Fig 1, data in S5 Table).

315 **Fig 1. PRV RNA levels in blood and heart.** PRV RNA levels (Ct values) in blood (A) and

316 heart (B) from naïve fish sampled at Day 0 (white dots), non-infected controls (Ctrl, grey dots),

317 PRV-infected (PRV, black dots) and PRV-infected fish exposed to periodic hypoxic stress

318 (PRV-H, red dots), at each time-point during the infection trial. Weeks post-infection (WPI)

319 are indicated on the x-axis. Ct value $\geq$ 37.0 indicates no virus RNA detected. A non-parametric

320 Mann-Whitney unpaired rank test was performed between the groups at all time-points.

321

322 Histopathological changes consistent with HSMI [46,47] including epicarditis, myocardial

323 mononuclear cell infiltration and myocardial necrosis were detected from 7 WPI in both

324 infected groups (Fig 2). Pathological changes were scored on a continuous visual analogue scale

325 separately in three compartments of the heart (i.e. epicardium, compactum and the spongious

326 (trabecular) layers). The averaged sum of scores for all compartments was calculated as the

327 total HSMI score (Fig 2A). In both groups, total HSMI score peaked at 10 WPI and declined

328 until 15 WPI. There were no significant differences in either total HSMI score or separate scores

329   in each compartment between PRV and PRV-H groups (Fig 2A-D). However, the PRV-H group

330   tended to have a stronger reduction in total score levels and compactum and spongiosum scores

331   from peak levels (10 WPI) until 12 WPI compared to the PRV group (Fig 2A, C and D).

332   **Fig 2**. **Histological scoring of inflammatory changes in the heart.** Development of

333   inflammatory changes is displayed for each group throughout the challenge trial. Ctrl: non-

334   infected controls, PRV: PRV-infected fish, PRV-H: PRV-infected fish exposed to periodic

335   hypoxic stress. Inflammatory changes in epicardium, compactum and spongiosum were scored

336   from sections of the heart ventricle using a continuous visual analogue scale ranging from 0-3.

337   The total HSMI score was calculated from the mean of scores from the separate heart

338   compartments. The lower and upper border of boxes indicates the 25th and 75th percentiles,

339   respectively and the centerline indicates the 50th percentile. The upper and lower whiskers

340   correspond to the highest and lowest value of the 1.5*IQR (inter-quartile range). A non-

341   parametric Mann-Whitney unpaired rank test was performed between the groups at all time-

342   points. Weeks post-infection (WPI) are indicated on the x-axis.

343

344   **Hypoxia tolerance is reduced during HSMI and improved by repeated hypoxic stress**

345   To test the hypothesis that PRV-infection and HSMI-related cardiomyopathy affects hypoxia

346   tolerance, an acute hypoxia challenge test (HCT) [48] was performed in common garden

347   experiments including 30 fish from each group at 4, 7 and 10 WPI. Time-course of water

348   oxygenation and temperature for each HCT are shown in S6 Fig. Cumulative incipient lethal

349   oxygen saturation (ILOS) levels for each group and time-point are plotted as Kaplan-Meier (K-

350   M) duration curves (Fig 3). Average values for ILOS and time (minutes) to cull 50 % of the test

351   groups (T_{50}) at each time-point are displayed in Table 2.

352   **Fig 3. Kaplan-Meier plots of tolerance time during hypoxia challenge test.** Percent

353   cumulative incipient lethal oxygen saturation (ILOS) levels over time during acute hypoxia

challenge of the Ctrl (grey line), PRV (black line) and PRV-H (red line) groups at 4 (A), 7 (B) and 10 (C) weeks post-infection (WPI). Secondary y-axis and dotted line (blue) shows water oxygen levels (% air saturation). Statistical significance levels are indicated in the bottom left of each plot after performing a Peto & Peto modification of the Gehan-Wilcoxon test between the curves for each group; Ctrl vs PRV (4, 7 and 10 WPI), Ctrl vs PRV-H (7 and 10 WPI) and PRV-H vs PRV (10 WPI). NS indicates not significant.

Table 2. Measures from the hypoxia challenge test.

	4 WPI		7 WPI		10 WPI	
	ILOS	*T_{50}*	*ILOS*	*T_{50}*	*ILOS*	*T_{50}*
Ctrl	20.32 ± 0.17^a	175	19.26 ± 0.61^a	127	17.86 ± 0.33^a	115
PRV	19.74 ± 0.16^a	222	20.41 ± 0.15^a	113	19.32 ± 0.16^b	98
PRV-H			19.99 ± 0.21^a	113	18.36 ± 0.30^a	109

Average ILOS levels ($\pm$ SE) and time (min) to cull 50% of the experimental population (T_{50}) for each group (N=30) during HCT performed at 4, 7 and 10 weeks post-infection (WPI). Groups are non-infected controls (Ctrl), infected (PRV) and infected exposed to periodic hypoxic stress (PRV-H). Statistical differences ($p < 0.05$) are indicated by superscript letters.

At 4 WPI (prior to first hypoxic stress treatment), infected fish were more hypoxia tolerant than Ctrl according to K-M curves ($p < 0.05$; Fig 3A) and T_{50} values (222 versus 175, respectively; Table 2). However, there were no group differences with regard to average ILOS levels (Table 2). At 7 WPI, when PRV RNA levels peaked and heart pathology was pronounced, both infected groups were less hypoxia tolerant than Ctrl according to K-M curves ($p < 0.0001$; Fig 3B) and T_{50} values (113 versus 127; Table 2). Average ILOS for PRV and PRV-H groups were not significantly different from, but numerically higher than the Ctrl group (Table 2). At 10

WPI, when heart pathology reached peak levels, K-M curves showed that the PRV group was less hypoxia tolerant compared to both the Ctrl ($p < 0.0001$) and the PRV-H ($p < 0.001$) group (Fig 3C). ILOS and T_{50} values were also lower for the PRV group (19.32 / 98) compared to the Ctrl (17.86 / 115) and the PRV-H (18.36 / 109; Table 2) group. There were no differences in PRV RNA levels (blood and heart) between infected individuals selected for the HCT at any time-point (Fig 4).

Fig 4. PRV RNA levels – HCT. PRV RNA levels (Ct values) in blood (A) and heart (B) from fish included in the HCT. Groups are non-infected controls (Ctrl, grey dots), infected (PRV, black dots) and infected exposed to periodic hypoxic stress (PRV-H, red dots). A non-parametric Mann-Whitney unpaired rank test was performed between the groups at all time-points. Weeks post-infection (WPI) are indicated on the x-axis.

The histopathological scoring showed a trend of higher inflammation score in all heart compartments for the PRV-H group compared to the PRV-infected group (Fig 5). Histopathological evaluation of gill sections collected from all fish included in the HCT showed no pathological lesions (data not shown).

Fig 5. Histological scoring of inflammatory changes in the heart – HCT. Scoring of the inflammatory changes for the fish included in the HCT are displayed by groups. Ctrl: non-infected control group, PRV: PRV-infected group, PRV-H: PRV-infected exposed to periodic hypoxic stress. Inflammatory changes in epicardium, compactum and spongiosum were scored from sections of the heart ventricle using a continuous visual analogue scale ranging from 0-3. The total HSMI score was calculated from the mean of scores from the separate heart compartments. The lower and upper border of boxes indicates the 25th and 75th percentiles, respectively and the centerline indicates the 50th percentile. The upper and lower whiskers

398 correspond to the highest and lowest value of the 1.5*IQR (inter-quartile range). A non-

399 parametric Mann-Whitney unpaired rank test was performed between the groups at all time-

400 points. Weeks post-infection (WPI) are indicated on the x-axis.

401

402 **HSMI-related cardiomyopathy reduces optimum temperature (T_{opt}) for aerobic scope,**

403 **which is improved after repeated hypoxic stress**

404 In order to evaluate if cardiac histopathological changes associated with HSMI and additional

405 periodic hypoxic stress affect specific measures of cardiac performance and thermal tolerance,

406 maximum heart rate (f_{Hmax}) measurements were performed at 10 WPI, when histopathological

407 changes were most pronounced. Infected fish included in the analysis had similar levels of

408 histopathological changes in the heart (data in S7 Fig). There were no group differences in f_{Hmax}

409 during the linear phase (12-18 °C), but PRV-infected fish had lower f_{Hmax} at 19 °C (Fig 6A).

410 Average f_{Hmax} between 13-19 °C showed that the PRV group had respectively 5.7 and 6.1 lower

411 f_{Hmax} than the PRV-H and Ctrl ($p > 0.05$) groups (Fig 6B). A two-way ANOVA showed that

412 the PRV group had a lower heart rate (-4.7 BPM, $p = 0.07$) than the other two groups (Fig 6B).

413 T_{opt} for aerobic scope was determined from Arrhenius breakpoint temperature calculations for

414 f_{Hmax} (Fig 6C), showing a higher T_{opt} for the Ctrl (16 °C) group compared to the PRV-H (15.3

415 °C) and the PRV (14.7 °C) groups.

416 **Fig 6. Maximum heart rate measurements**. Maximum heart rate (f_{Hmax}) measurements at 10

417 WPI in non-infected controls (Ctrl, grey line), PRV-infected (PRV, black line) and PRV-

418 infected exposed to periodic hypoxic stress (PRV-H, red line). A: Average f_{Hmax} (±SE) during

419 temperature increase for each group. Dashed lines between dots indicate that half or more

420 individuals of the initial population were missing or had cardiac arrhythmia and therefore were

421 removed from the measurement. Grey areas and asterisks indicate significant differences

422 between groups (ANOVA, *: $p < 0.05$). Point "acc" shows f_{Hmax} after acclimation at 12 °C, just

423 before atropine injection. B: Average f_{Hmax} (±SE) of the three groups between 13 and 19 °C. C:

424 T_{opt} for aerobic scope calculated from Arrhenius breakpoint temperature of f_{Hmax} for each group.

425 No significant correlation (Pearson's product-moment correlation) between heart rate and virus

426 RNA level was found (r = 0.15, p = 0.51) (data in S8A Fig). The linear model indicated a

427 decreased f_{Hmax} with increasing virus RNA level. However, this effect was weak with 0.89

428 decrease in f_{Hmax} per virus Ct value (i.e. doubling of virus RNA transcripts) (data in S8B Fig).

429

430 **PRV infection combined with periodic hypoxic stress reduces blood oxygen affinity**

431 The effect of PRV-infection and periodic hypoxic stress on blood oxygen affinity was evaluated

432 from Hb-oxygen dissociation curves (ODC) calculated for PRV-H and Ctrl groups at 10 WPI

433 (Fig 7). Measurements were corrected for Hb concentrations. Average Hb levels for Ctrl and

434 PRV-H were respectively 12.2 and 10.9 g/100 ml (p > 0.05; Fig 8), and average ATP levels

435 0.63 and 0.74 nmol/µl (p = 0.07; Fig 7A, data in S9 Fig). There was an apparent right-shift of

436 the ODC and resulting increased P_{50} for the PRV-H group (49.7 mm Hg) compared with the

437 Ctrl group (29.5 mm Hg) (Fig 7A). Maximal oxygen saturation was also lower in the PRV-H

438 group compared with the controls (Fig 7A). Linear regression analysis of ODC data also

439 showed a higher Hill coefficient and K_d value for the Ctrl versus the PRV-H group (Fig 7B).

440 **Fig 7. Blood oxygen binding affinity.** Blood oxygen binding affinity in PRV-infected fish

441 exposed to periodic hypoxic stress (PRV-H, red curve/line) and non-infected controls (Ctrl,

442 black curve/line) at 10 weeks post-infection (WPI). A: Hb-O_2 dissociation curves (ODC)

443 relating partial pressure of oxygen (P_{O2}; x-axis) with Hb-oxygen saturation (y-axis). ATP

444 concentrations are shown in inset with significance level (p = 0.07, indicated by +) according

445 to non-parametric Mann-Whitney unpaired rank test. B: Linear regression of ODC from log-

446 transformed data showing Hill coefficients and K_d (zero intercept) values for the groups.

447

Pearson correlation analysis showed positive correlation between PRV Ct values in heart and Hb concentration (r = 0.41, $p < 0.001$) at 7 and 10 WPI (N = 71, Ctrl group omitted). A linear regression analysis showed that for every increase in Hb concentration in blood, the Ct value of PRV RNA in heart increases with 0.28 (adj. $r^2 = 0.15$, $p < 0.01$) (Fig 8, data in S10A Fig). PRV Ct values in blood showed a lower degree of correlation to Hb (r = 0.22, $p < 0.05$) (N = 88), however the regression analysis did not detect any effects of PRV Ct values in blood (data in S10B Fig).

Fig 8. Hemoglobin levels and correlations with PRV Ct values. A: Hemoglobin (Hb) concentration (y-axis) in non-infected controls (Ctrl, grey), PRV-infected (PRV, black) and PRV-infected exposed to periodic hypoxic stress (PRV-H, red). The lower and upper border of boxes indicates the 25th and 75th percentiles, respectively and the centerline indicates the 50th percentile. The upper and lower whiskers correspond to the highest and lowest value of the 1.5*IQR (inter-quartile range). Significance is indicated by * with a $p < 0.05$ and + with a $p = 0.07$. B: Pearson correlation analysis and linear regression analysis of PRV Ct values in heart (x-axis) and hemoglobin concentrations (y-axis). The analysis was performed on merged data-points from 7 (black dots) and 10 (red dots) weeks post infection (WPI). The correlation coefficient (r) with p value and linear regression output with the regression line in blue (95 % CI is shaded gray) and p value is stated.

466

Discussion

468 In the present study, we found that both hypoxia tolerance and cardiac performance are

469 impaired during peak levels of PRV-infection and HSMI lesions in Atlantic salmon. Repeated

470 short-term hypoxic stress treatments during disease development diminished these effects.

471 The hypoxia tolerance challenge test was performed at early viral replication prior to HSMI

472 specific lesions (4 WPI), peak virus load and initial lesions (7 WPI), and peak levels of lesions

473 (10 WPI). Contrary to our expectations, the HCT performed at 4 WPI showed a significantly

474 higher hypoxia tolerance for the infected group compared to the controls. The PRV-infected

475 group had also significantly higher weight compared to the Ctrl group. Increased body mass

476 has variable effect on hypoxia tolerance in fish [48–50], however we found no effect of body

477 weight on ILOS, either across all time-points (all data compiled) or at separate time-points.

478 Furthermore, at 4 WPI, 70 % of the infected individuals had moderate levels of viral RNA in

479 blood cells. The improved hypoxia tolerance could be related to a priming response caused by

480 the infection in these cells. For example, a recent study by Johansen et al. [51] showed

481 upregulation of genes related to iron metabolism and erythropoiesis four weeks after PRV

482 infection. However, this should be reflected by increased levels of hemoglobin, which was not

483 found here. In a similar PRV study, induced erythrocytic transcription of catalase,

484 glucocorticoid receptor and hypoxia-inducible transcription factor (HIF-1α2) was observed

485 [22]. These genes have important roles in responses to tissue hypoxia in higher vertebrates

486 [52,53]. Hence, PRV-infection of erythrocytes induces genes that may increase the hypoxia

487 tolerance.

488 At 7 WPI, when PRV RNA levels peaked and histopathological lesions were developing, the

489 PRV-infected groups were less hypoxia tolerant compared to non-infected controls. Previously,

490 the transcription of innate antiviral genes are shown to be strongly induced at this stage of the

491 PRV-infection [16,22,51]. The viral transcription, subsequent innate antiviral immune response

492 in erythrocytes and the onset of myocardial inflammation may contribute to the impaired

493   hypoxia tolerance. The group exposed to periodic hypoxic stress (PRV-H; 4 h of 40 % O_2)

494   intended to simulate incidences of hypoxia in the field, i.e. during handling operations or natural

495   reduction in water exchange during the turn of tidal currents [27,54], did not show any

496   difference in performance from the PRV group. This implies that a single previous treatment

497   with hypoxic stress (at 4 WPI) did not affect the hypoxia tolerance, which is in line with

498   previous reports showing that salmonids are adapted to physiologically and metabolically

499   counteract transient low oxygen levels [32,55].

500   The reduced hypoxia tolerance was still evident three weeks later (10 WPI) for the PRV group,

501   but not for the PRV-H group. There was no difference in PRV RNA levels or HSMI scores

502   between the two infected groups at this time-point. The only difference between the infected

503   groups was the two previous hypoxic stress treatments of the PRV-H group, which may indicate

504   a stress-conditioning effect [56,57]. However, this should be followed up in future studies also

505   including non-infected fish subjected to the same treatment.

506   The maximum heart rate measurements at 10 WPI were in line with the reduced hypoxia

507   tolerance observed for the PRV group. This group had lower f_{Hmax} compared to the PRV-H and

508   Ctrl groups, which was significant at 19 °C. Furthermore, the T_{opt} for aerobic scope was lower

509   supporting that infection and/or inflammation impaired cardiac capacity. The lower f_{Hmax} and

510   T_{opt} observed for the PRV group may be due to reflex bradycardia, which is a common cardiac

511   response to hypoxia in fish [31]. A regression analysis did not detect any impact of individual

512   virus load (PRV Ct values) on the f_{Hmax} between PRV and PRV-H groups, implying that hypoxic

513   stress treatment rather than the viral load was explaining differences in performance.

514   Despite similar cardiac performance and hypoxia tolerance to the Ctrl group at 10 WPI, the

515   PRV-H group had a reduced blood oxygen binding affinity. The improved cardiac performance

516   and higher aerobic scope for the PRV-H group may have compensated for the reduced blood

517   oxygen affinity. However, this remains speculative since blood oxygen affinity was not

518   measured in the PRV group. Our results highlight the possible negative consequences of

519   erythrocyte infection and cardiac inflammatory lesions on the cardiorespiratory performance of

520   salmonids. The finding that short-term environmental hypoxic stress may improve tolerance to

521   secondary acute hypoxia in post-smolts is intriguing.

522   The mean incipient lethal oxygen saturation measured in the current study are considerably

523   higher than previously published for salmonids [48,58,59]. This discrepancy may be due to

524   differences in experimental protocol, water salinity and temperature and life stage of the

525   experimental fish [60]. The experimental fish in our study were reared in brackish water and

526   challenged with the first HCT six weeks after transfer from freshwater. The period before and

527   after seawater transfer is considered to be challenging and energy consuming for salmonids.

528   The increased metabolic and physiological demands after seawater transfer may reduce the

529   capacity to handle hypoxic environment. This may reduce the hypoxic tolerance and hence

530   explain the higher ILOS values, as observed in our trial. Variations in hypoxia tolerance

531   between strains of rainbow trout [48] and between individuals of Atlantic salmon [39,59] may

532   also explain differences in observed ILOS between studies. A size or age dependent reduction

533   in T_{50} and ILOS as the trial proceeded may be present in our study, although no firm causality

534   between these factors has been detected in previous comparable studies [48,49,61]. Another

535   likely explanation was the fact that the biomass increased from the first to the last HCT as the

536   fish grew bigger. Despite minor differences in the levels of critical oxygen saturation between

537   groups, the results strongly suggested that HSMI is associated with reduced hypoxia tolerance,

538   reflected by the shorter time to loss of equilibrium (K-M curves for ILOS and T_{50}).

539   Scoring of histopathological changes in heart revealed no major differences in HSMI

540   development between the infected groups. The myocytes in the spongious layer of the salmonid

541   heart ventricle are passively oxygenated by the venous blood entering the lumen of the

542   ventricle. This is in contrast to the oxygenated blood from the gills supplying the compactum

543   via the coronary circulation [31,32]. The blood flow in the coronary arteries has been shown to

544   increase during hypoxia and exercise in rainbow trout and Coho salmon [62,63]. The

545   subsequent effect on inflammatory development due to the difference in oxygen supply of the

546   different myocardial layers remains to be investigated. Our findings did not detect any

547   differences in inflammatory severity between these strata of the heart. However, this

548   mechanism cannot be excluded on the basis of our challenge trial due to the time separating the

549   sampling points.

550   At 12 WPI, when the PRV-H group had experienced three episodes of hypoxic treatment, this

551   group tended to have lower histopathology scores in the heart, indicating a better myocardial

552   recovery for these individuals. Few studies have investigated the consequences of hypoxia on

553   disease development following a viral infection in teleosts. In SPDV challenged Atlantic

554   salmon post-smolts, long-term hypoxia at 12 °C ($60 - 65$ % O_2 saturation for 70 days) had no

555   effect on pancreas disease development [30]. Another study investigated the immune responses

556   during chronic hypoxia in Atlantic salmon post-smolts stimulated with poly I:C or a water-

557   based *Vibrio anguillarum* vaccine [64]. The fish were exposed to 58 days of hypoxia (52 % O_2

558   saturation at 10.5 °C) and the results showed a reduced expression of immune related genes, *in*

559   *vitro* and *in vivo* [64], potentially connected to hypoxia-induced production of corticosteroids

560   such as cortisol [29,64]. Such responses may repress the innate and adaptive immune responses

561   during viral infection and hence lower the degree of inflammation within infected tissues. In

562   zebrafish, hypoxia induced responses have been shown to improve myocardial regeneration

563   [65,66]. In sum, this represents putative explanations to why the PRV-H group had reduced

564   levels of inflammation and heart lesions at 12 WPI compared to the PRV group.

565   The three periodic hypoxic stress treatments during experimental PRV-infection did not affect

566   PRV RNA levels when compared to infected fish without hypoxic stress. Nevertheless, we

567   cannot rule out that hypoxia during earlier stages of infection may have a different effect on

568   virus levels. Such short-term environmental hypoxia may have effects on the integrity of barrier

569   defense organs (i.e. skin and gill). Therefore, hypoxic stress prior to and during the actual phase

570   of virus uptake from water should be addressed in future studies.

571

572   Measurements of Hb-oxygen affinity detected an apparent difference in P_{50} between the control

573   group and the PRV-H group suggesting a Bohr effect occurring in the latter group. To the

574   author's knowledge, this is the first report showing decreased Hb-oxygen affinity in viral-

575   infected erythrocytes in fish. Studies on rats infected with malaria (*Plasmodium berghei*

576   *berghei*) showed a similar reduced Hb-oxygen affinity in infected erythrocytes at

577   intraerythrocytic pH $\leq$ 7.0 [67,68], detected as a right-shift of the ODC [67]. In the latter study,

578   ATP concentration in infected erythrocytes increased five-fold due to increased parasite

579   production, hence explaining the reduced oxygen affinity. Soivio *et al.* [69] detected an increase

580   in blood oxygen affinity in rainbow trout exposed to hypoxia for 12 days (25-35 % O_2, 11 °C).

581   The increase was detected within 6 h of hypoxia and reached steady state after 8 days of

582   hypoxia. A reduced ATP content of 50 % was detected in the erythrocytes of the hypoxic fish

583   after one week [69] and was suggested to increase the oxygen carrying capacity of Hb [33]. In

584   our study, ATP levels tended to be higher in the PRV-H group compared to non-infected

585   controls. This corresponded to a reduction in the oxygen carrying capacity of Hb in the PRV-

586   H group compared to the controls (right-shifted ODC, increased P_{50} and lower maximal oxygen

587   saturation). This apparent reduced oxygen carrying capacity (sometimes referred to as the Root

588   effect when caused by reduced intracellular pH) is a common feature of fish Hb that greatly

589   enhances oxygen delivery under exercise or hypoxia [44]. In the present study, fish

590   experiencing periodic hypoxia in combination with the presence of viral replication within red

591   blood cells would likely be a compensation to increase their tissue oxygen delivery.

592

Interestingly, the Hb concentrations were significantly reduced in both infected groups at 7 WPI, when reduced hypoxia tolerance was observed for both groups. In line with this, PRV RNA levels in heart correlated with Hb concentration for individuals at 7 and 10 WPI. Hemolytic anemia has been reported for a number of salmonid diseases such as viral hemorrhagic septicemia [70] and ISA [71]. Recently, a variant of PRV was demonstrated to be the causal agent of erythrocytic inclusion body syndrome (EIBS) in Coho salmon, a disease associated with anemia [12]. Although anemia is not a common finding in HSMI, the current data indicate that dips in hemoglobin may occur during the peak phase of PRV infection. However, in the linear model, PRV Ct values explained 15 % of the total variation in Hb concentration measured in PRV-infected individuals at 7 and 10 WPI.

The potential impact of PRV-infection and HSMI on hypoxia tolerance and cardiorespiratory performance observed in this study highlights the importance of further increasing our knowledge on this topic, in order to improve the fish health and prevent losses in Atlantic salmon aquaculture. Future research should focus on understanding the molecular and pathological mechanisms underlying the observed differences in hypoxia tolerance.

Acknowledgements

Authors' contributions

MKD, VA and ER launched the project idea. SMJ and MKD participated in the overall design and coordination of the study, interpretation of data and drafting the manuscript. SMJ performed and interpreted the data from HCT. MP performed and interpreted the data from the

hemoglobin-oxygen dissociation measurements and drafted the manuscript. GT performed and

interpreted the data from the maximum heart rate measurement and drafting the manuscript.

MA performed histopathological scoring of heart sections and revised the manuscript. ER and

VA participated in the coordination of the study and revised the manuscript. ML participated

in the overall challenge design, coordinated the study, all practicalities regarding sample

logistics, assisting in performing the HCT, gathering and interpretation of data and drafted the

manuscript. ML performed the histopathological scoring of heart sections, and Hb and ATP

measurements. All authors read and approved the final manuscript.

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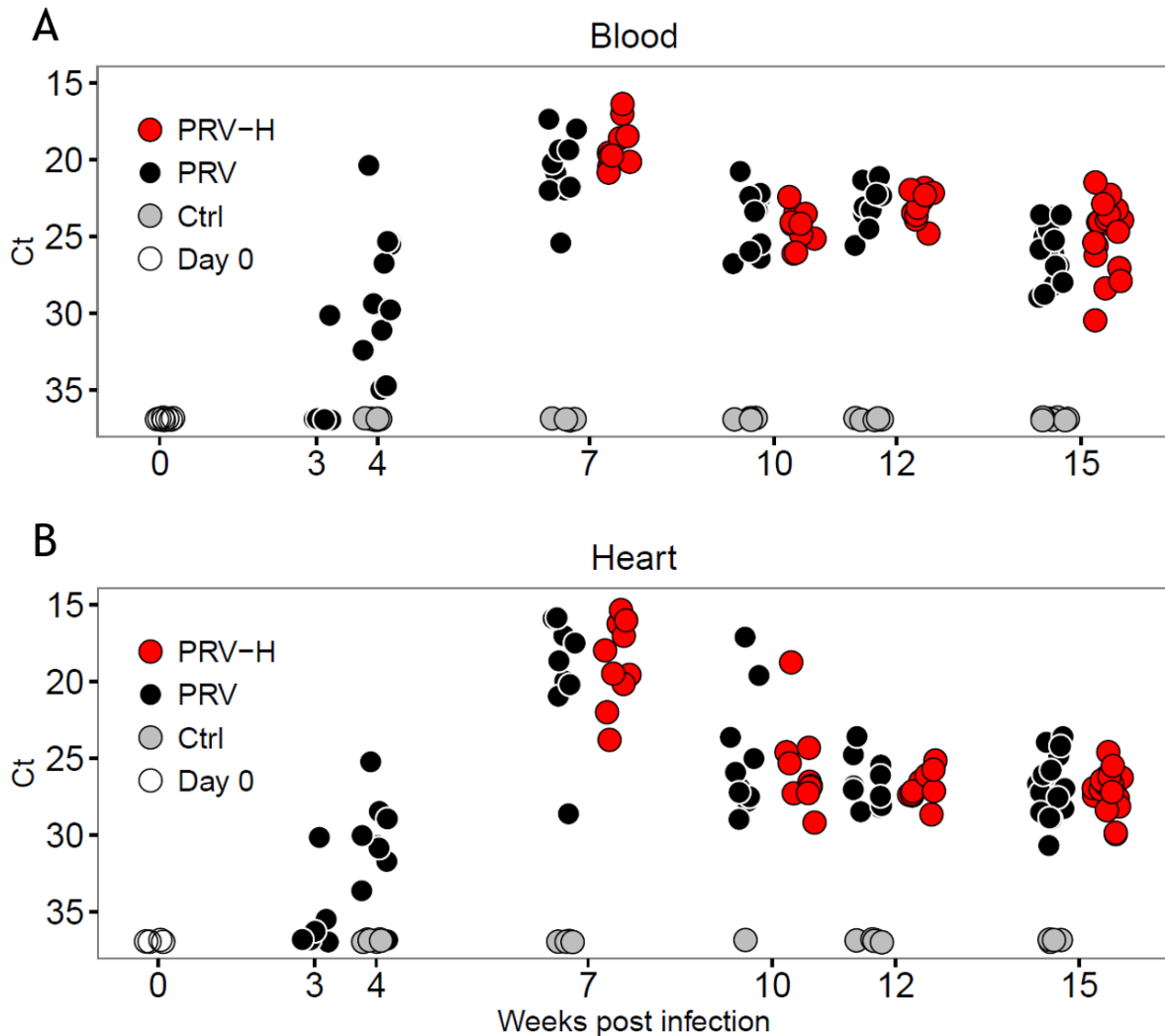
Figures

Fig. 1

Fig 1. PRV RNA levels in blood and heart. PRV RNA levels (Ct values) in blood (A) and heart (B) from naïve fish sampled at Day 0 (white dots), non-infected controls (Ctrl, grey dots), PRV-infected (PRV, black dots) and PRV-infected fish exposed to periodic hypoxic stress (PRV-H, red dots), at each time-point during the infection trial. Weeks post-infection (WPI) are indicated on the x-axis. Ct value ≥ 37.0 indicates no virus RNA detected. A non-parametric Mann-Whitney unpaired rank test was performed between the groups at all time-points.

851   **Fig. 2**
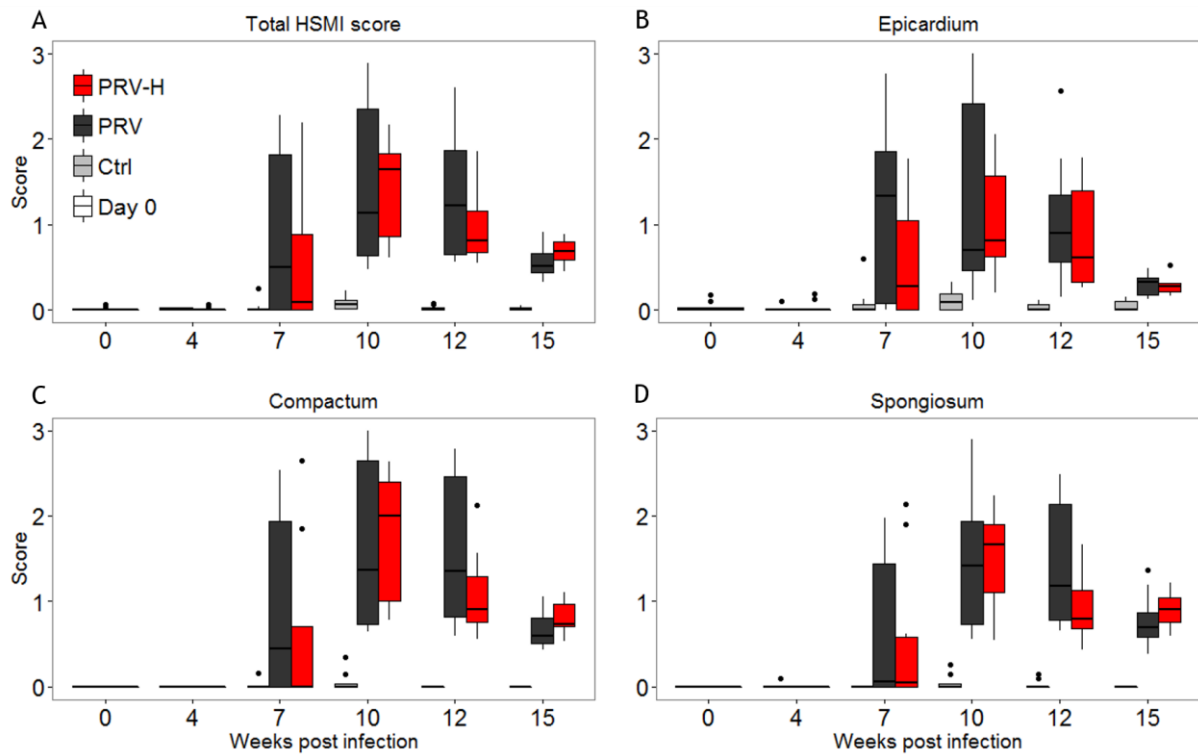
852

853   **Fig 2**. **Histological scoring of inflammatory changes in the heart.** Development of inflammatory
854   changes is displayed for each group throughout the challenge trial. Ctrl: non-infected controls, PRV:
855   PRV-infected fish, PRV-H: PRV-infected fish exposed to periodic hypoxic stress. Inflammatory
856   changes in epicardium, compactum and spongiosum were scored from sections of the heart ventricle
857   using a continuous visual analogue scale ranging from 0-3. The total HSMI score was calculated from
858   the mean of scores from the separate heart compartments. The lower and upper border of boxes
859   indicates the 25th and 75th percentiles, respectively and the centerline indicates the 50th percentile. The
860   upper and lower whiskers correspond to the highest and lowest value of the 1.5*IQR (inter-quartile
861   range). A non-parametric Mann-Whitney unpaired rank test was performed between the groups at all
862   time-points. Weeks post-infection (WPI) are indicated on the x-axis.

863   **Fig. 3**
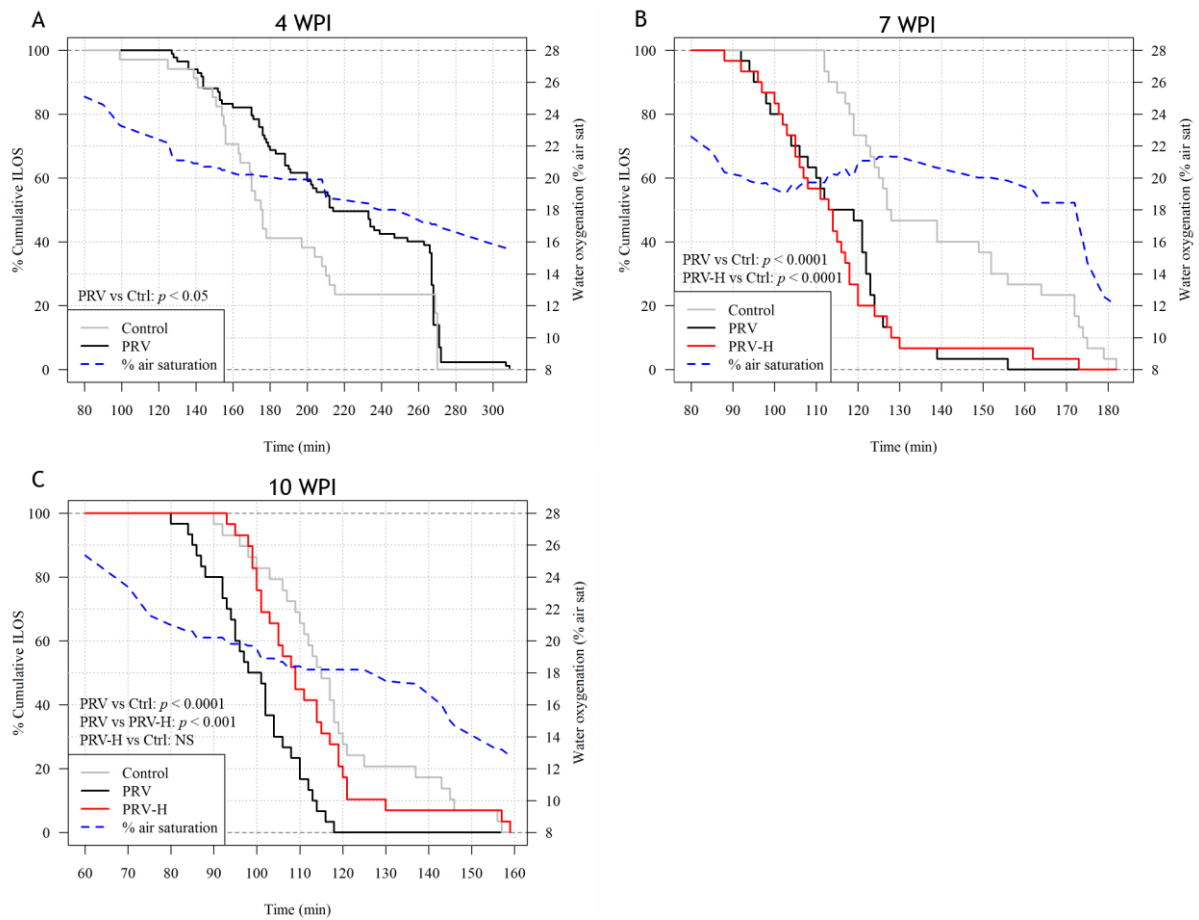
864

865   **Fig 3. Kaplan-Meier plots of tolerance time during hypoxia challenge test.** Percent cumulative

866   incipient lethal oxygen saturation (ILOS) levels over time during acute hypoxia challenge of the Ctrl

867   (grey line), PRV (black line) and PRV-H (red line) groups at 4 (A), 7 (B) and 10 (C) weeks post-

868   infection (WPI). Secondary y-axis and dotted line (blue) shows water oxygen levels (% air saturation).

869   Statistical significance levels are indicated in the bottom left of each plot after performing a Peto &

870   Peto modification of the Gehan-Wilcoxon test between the curves for each group; Ctrl vs PRV (4, 7

871   and 10 WPI), Ctrl vs PRV-H (7 and 10 WPI) and PRV-H vs PRV (10 WPI). NS indicates not

872   significant.

873 **Fig. 4**

Fig 4. PRV RNA levels – HCT. PRV RNA levels (Ct values) in blood (A) and heart (B) from
fish included in the HCT. Groups are non-infected controls (Ctrl, grey dots), infected (PRV,
black dots) and infected exposed to periodic hypoxic stress (PRV-H, red dots). A non-
parametric Mann-Whitney unpaired rank test was performed between the groups at all time-
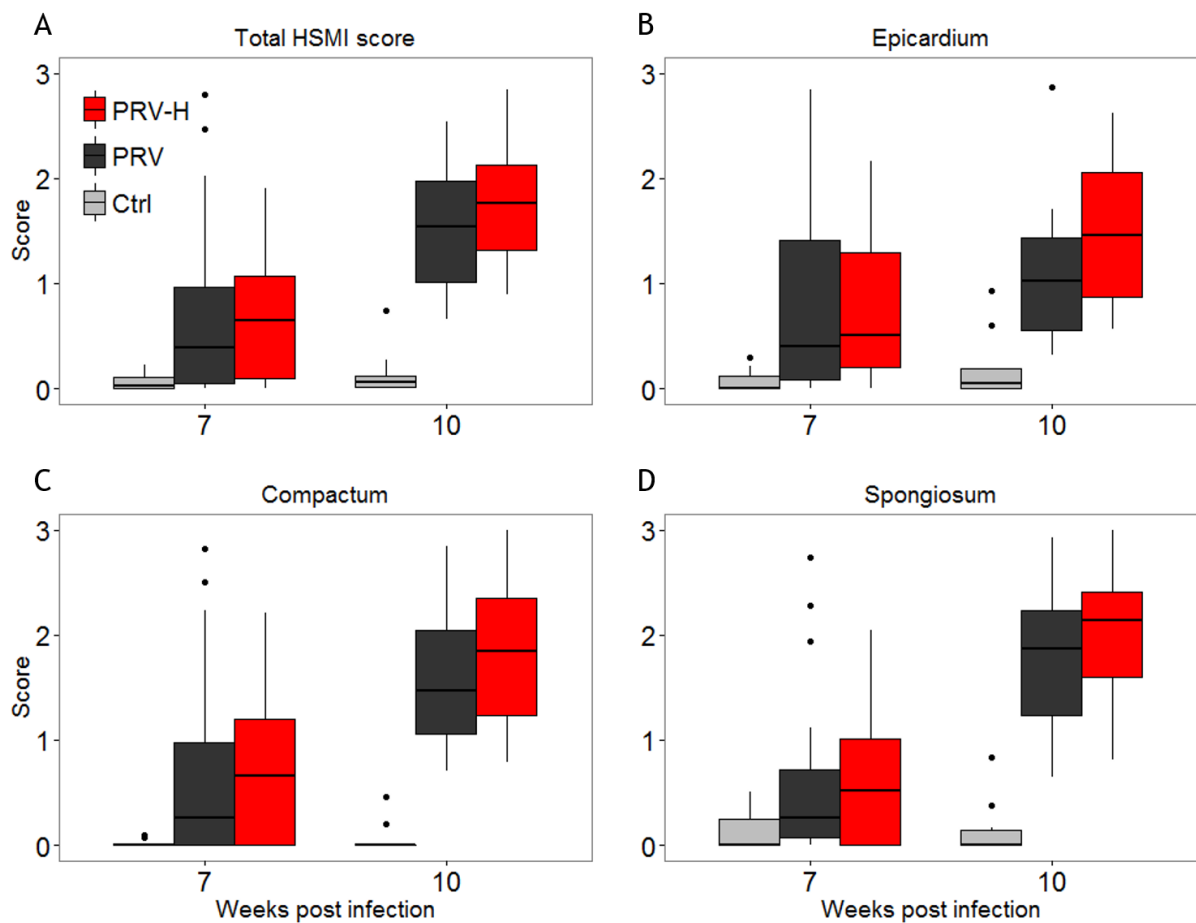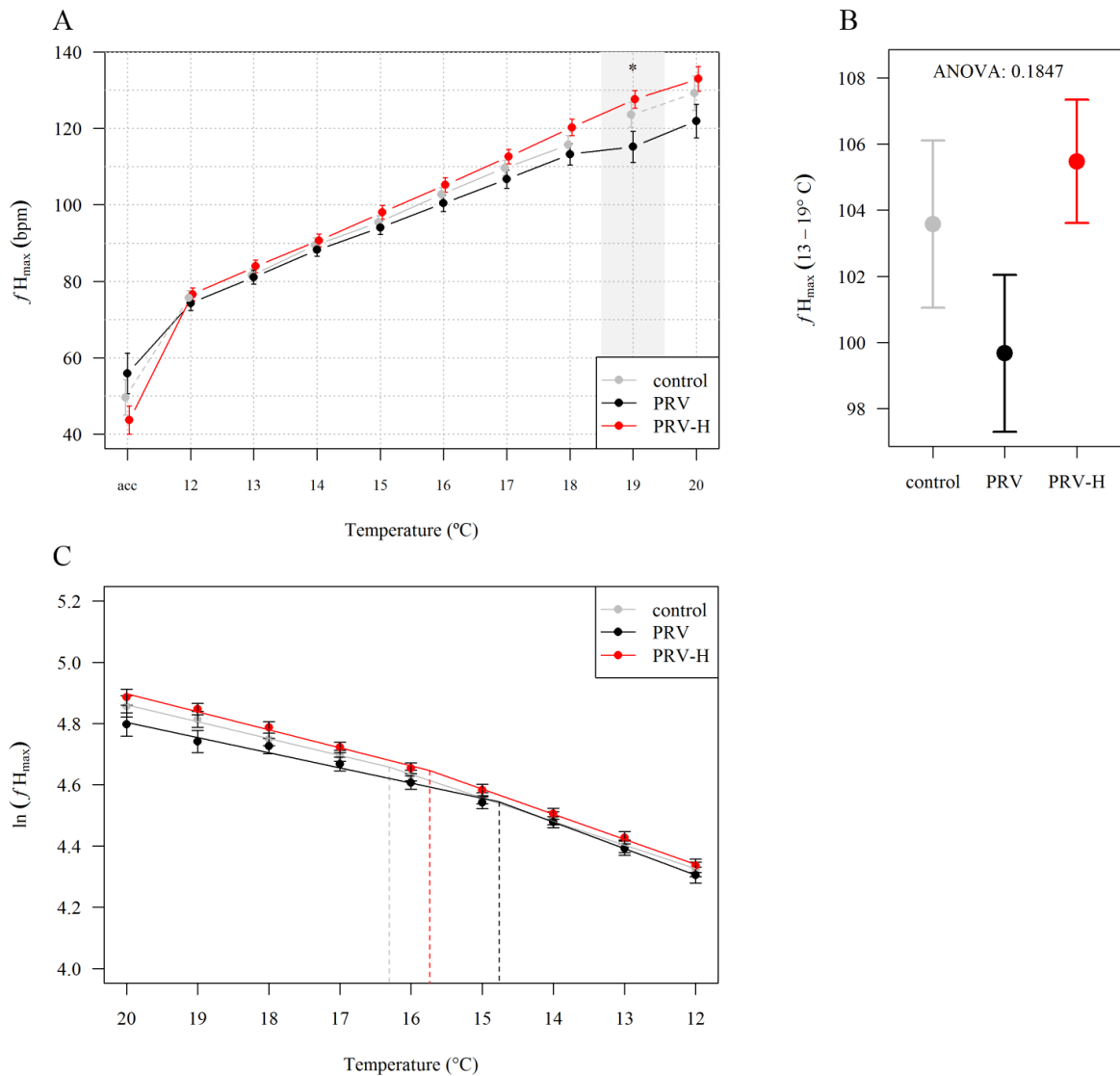points. Weeks post-infection (WPI) are indicated on the x-axis.

Fig. 5

Fig 5. Histological scoring of inflammatory changes in the heart – HCT. Scoring of the inflammatory changes for the fish included in the HCT are displayed by groups. Ctrl: non-infected control group, PRV: PRV-infected group, PRV-H: PRV-infected exposed to periodic hypoxic stress. Inflammatory changes in epicardium, compactum and spongiosum were scored from sections of the heart ventricle using a continuous visual analogue scale ranging from 0-3. The total HSMI score was calculated from the mean of scores from the separate heart compartments. The lower and upper border of boxes indicates the 25th and 75th percentiles, respectively and the centerline indicates the 50th percentile. The upper and lower whiskers correspond to the highest and lowest value of the 1.5*IQR (inter-quartile range). A non-parametric Mann-Whitney unpaired rank test was performed between the groups at all time-points. Weeks post-infection (WPI) are indicated on the x-axis.
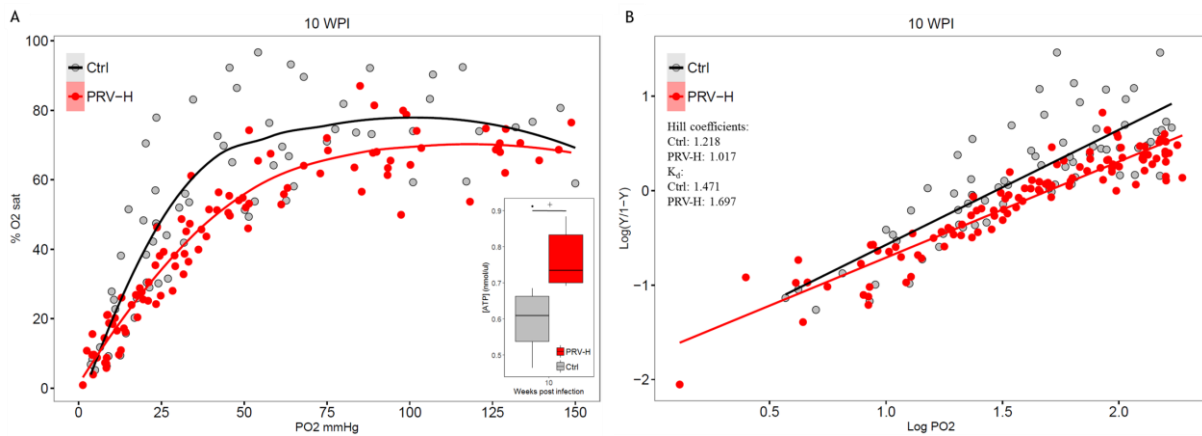
Fig. 6

A

B

C

Fig 6. Maximum heart rate measurements. Maximum heart rate (f_{Hmax}) measurements at 10 WPI in non-infected controls (Ctrl, grey line), PRV-infected (PRV, black line) and PRV-infected exposed to periodic hypoxic stress (PRV-H, red line). A: Average f_{Hmax} (±SE) during temperature increase for each group. Dashed lines between dots indicate that half or more individuals of the initial population were missing or had cardiac arrhythmia and therefore were removed from the measurement. Grey areas and asterisks indicate significant differences between groups (ANOVA, *: $p < 0.05$). Point "acc" shows f_{Hmax} after acclimation at 12 °C, just before atropine injection. B: Average f_{Hmax} (±SE) of the three groups between 13 and 19 °C. C: T_{opt} for aerobic scope calculated from Arrhenius breakpoint temperature of f_{Hmax} for each group.

Fig. 7

Fig 7. Blood oxygen binding affinity. Blood oxygen binding affinity in PRV-infected fish exposed to periodic hypoxic stress (PRV-H, red curve/line) and non-infected controls (Ctrl, black curve/line) at 10 weeks post-infection (WPI). A: Hb-O_2 dissociation curves (ODC) relating partial pressure of oxygen (P_{O2}; x-axis) with Hb-oxygen saturation (y-axis). ATP concentrations are shown in inset with significance level ($p = 0.07$, indicated by +) according to non-parametric Mann-Whitney unpaired rank test. B: Linear regression of ODC from log-transformed data showing Hill coefficients and K_d (zero intercept) values for the groups.

Fig. 8

Fig 8. Hemoglobin levels and correlations with PRV Ct values. A: Hemoglobin (Hb) concentration (y-axis) in non-infected controls (Ctrl, grey), PRV-infected (PRV, black) and PRV-infected exposed to periodic hypoxic stress (PRV-H, red). The lower and upper border of boxes indicates the 25th and 75th percentiles, respectively and the centerline indicates the 50th percentile. The upper and lower whiskers correspond to the highest and lowest value of the 1.5*IQR (inter-quartile range). Significance is indicated by * with a $p < 0.05$ and + with a $p = 0.07$. B: Pearson correlation analysis and linear regression analysis of PRV Ct values in heart (x-axis) and hemoglobin concentrations (y-axis). The analysis was performed on merged data-points from 7 (black dots) and 10 (red dots) weeks post infection (WPI). The correlation coefficient (r) with p value and linear regression output with the regression line in blue (95 % CI is shaded gray) and p value is stated.

Supporting information

S1 Table. Scoring criteria for histopathological evaluation of heart sections.

Score	Criteria
Epicarditis	
0	No pathological changes observed
0.1 – 1.0	Focal or multifocal mononuclear infiltration of one cell layer in epicardium
1.1 – 2.0	Multifocal, moderate infiltration of 2-3 layers of inflammatory cells in epicardium
2.1 – 3.0	Extensive infiltration of >3 layers of inflammatory cells in epicardium
Myocardial inflammation in the compactum	
0	No pathological changes observed
0.1 – 1.0	Focal, mild infiltration of a limited number of mononuclear inflammatory cells
1.1 – 2.0	Focal to multifocal, moderate infiltration of mononuclear inflammatory cells
2.1 – 3.0	Diffuse and extensive infiltration of mononuclear inflammatory cells and myocardial necrosis
Myocardial inflammation in the spongiosum	
0	No pathological changes observed
0.1 – 1.0	Focal, mild infiltration of a limited number of mononuclear inflammatory cells
1.1 – 2.0	Focal to multifocal, moderate infiltration of mononuclear inflammatory cells
2.1 – 3.0	Diffuse and extensive infiltration of mononuclear inflammatory cells and myocardial necrosis

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933

934   **S2 Fig. Weight of the experimental fish.** All fish included in each group from Day 0 to 15

935   weeks post infection. Ctrl: non-infected controls, PRV: PRV-infected fish, PRV-H: PRV-infected fish

936   exposed to periodic hypoxic stress. The lower and upper border of boxes indicates the 25th and

937   75th percentiles, respectively and the centerline indicates the 50th percentile. The upper and

938   lower whiskers correspond to the highest and lowest value of the 1.5*IQR (inter-quartile range).

939   Significance is indicated by * with a $p < 0.05$.

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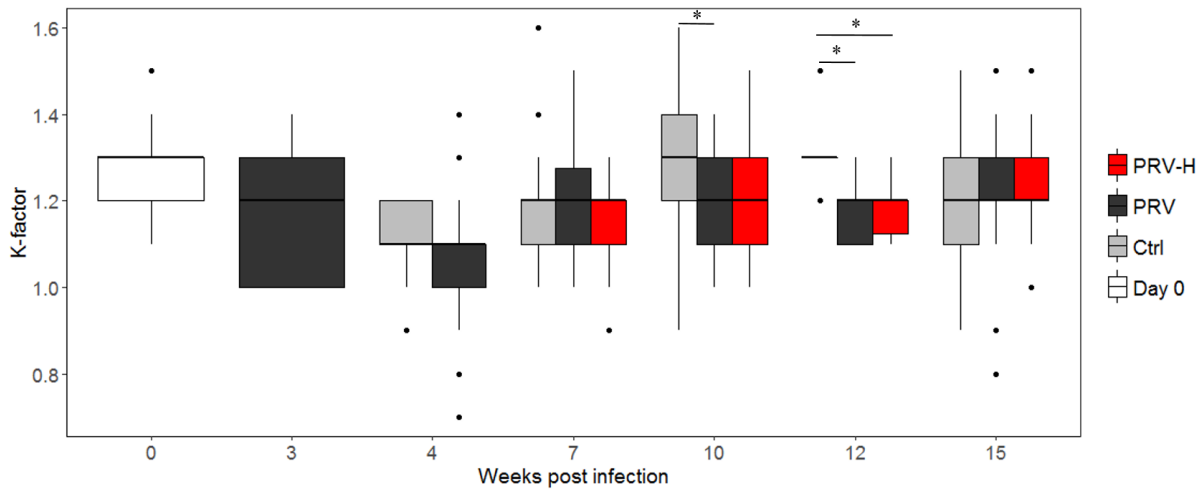
S3 Fig. K-factor of all fish in every group from Day 0 to 15 weeks post infection. Ctrl: non-
infected controls, PRV: PRV-infected fish, PRV-H: PRV-infected fish exposed to periodic
hypoxic stress. The lower and upper border of boxes indicates the 25th and 75th percentiles,
respectively and the centerline indicates the 50th percentile. The upper and lower whiskers
correspond to the highest and lowest value of the 1.5*IQR (inter-quartile range). Significance
is indicated by * with a $p < 0.05$.

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950

D
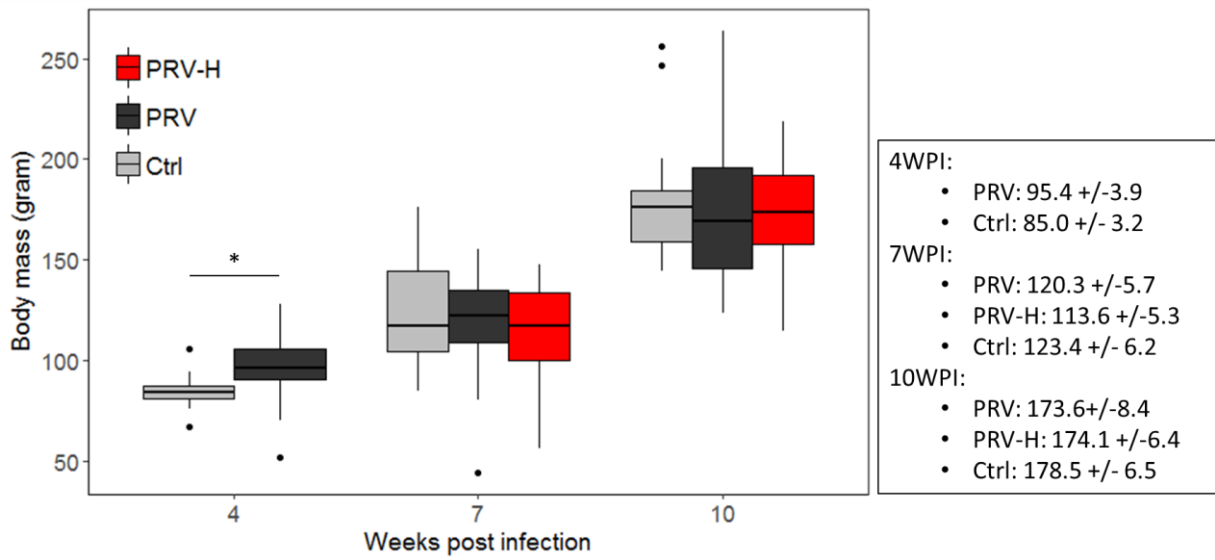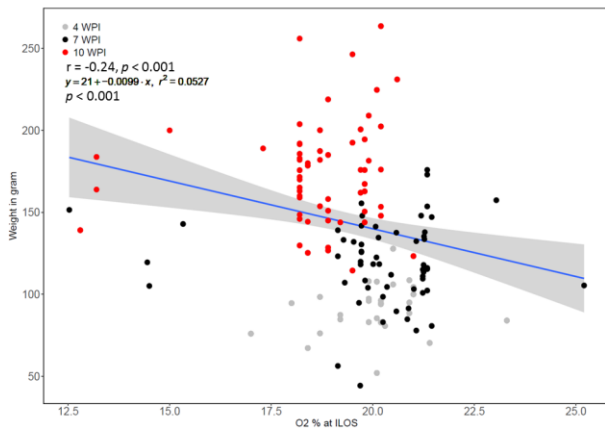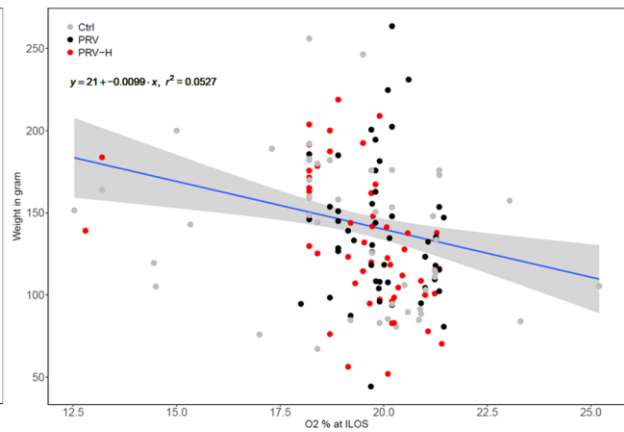
	Pearson correlation		lm(weight ~ O2%)	
	r	p	Adj. R^2	p
All data	-0.24	**	0.05	**
All data - 4 WPI	0.26	NS	0.03	NS
All data - 7 WPI	-0.05	NS	0.01	NS
All data - 10 WPI	0.12	NS	0.004	NS
Ctrl	-0.27	NS	0.05	NS
Ctrl - 4 WPI	0.44	NS	0.08	NS
Ctrl - 7 WPI	-0.08	NS	-0.05	NS
Ctrl - 10 WPI	-0.04	NS	-0.05	NS
PRV + PRV-H	-0.24	**	0.05	**
PRV + PRV-H - 4 WPI	0.22	NS	-0.002	NS
PRV + PRV-H - 7 WPI	0.04	NS	-0.02	NS
PRV + PRV-H - 10 WPI	0.18	NS	0.01	NS
PRV	-0.08	NS	-0.007	NS
PRV - 4 WPI	0.53	NS	0.19	NS
PRV - 7 WPI	0	NS	-0.05	NS
PRV - 10 WPI	0.36	*	0.1	*
PRV-H	-0.38	**	0.13	**
PRV-H - 4 WPI	0.24	NS	-0.04	NS
PRV-H - 7 WPI	0.02	NS	-0.05	NS
PRV-H - 10 WPI	0.12	NS	-0.01	NS

951

952 **S4 Fig. Relation between weight and oxygen saturation at ILOS.** A. Weight of the

953 individuals included in the hypoxia challenge test (HCT). Ctrl: non-infected controls, PRV:

954 PRV-infected fish, PRV-H: PRV-infected fish exposed to periodic hypoxic stress. The lower

955 and upper border of boxes indicates the 25th and 75th percentiles, respectively and the centerline

956 indicates the 50th percentile. The upper and lower whiskers correspond to the highest and lowest

957 value of the 1.5*IQR (inter-quartile range). Mean weight (+/-SEM) for each group at each time-

958 point is stated in the text box. Significance is indicated by * with a $p < 0.05$. B. Individual

959 weight plotted against the respective oxygen saturation at ILOS for all HCT's; 4 (grey dots), 7

960 (black dots) and 10 (red dots) weeks post infection (WPI) is indicated. The output of a linear

961 regression and Pearson correlation analysis is stated. C. Individual weight plotted against the

962 respective oxygen saturation at ILOS for all HCT's; Ctrl (grey dots), PRV (black dots) and

963 PRV-H (red dots) groups are indicated. The output of the same linear regression analysis as

964 performed in B is stated. D. Table of output from the linear regression and Pearson correlation

965   analysis for each group and separate HCTs performed at 4, 7 and 10 WPI. "lm(weight ~ O_2 %)"

966   indicate the linear regression model used.

967

968

WPI	0		3		4		7		10		12		15	
Group	Blood	Heart	Blood	Heart	Blood	Heart	Blood	Heart	Blood	Heart	Blood	Heart	Blood	Heart
PRV-H							16.3-20.9 (+/- 0.46)	15.3-23.8 (+/- 0.88)	22.3-26.3 (+/- 0.38)	18.9-29.3 (+/- 0.88)	21.8-24.7 (+/- 0.29)	25.0-28.7 (+/- 0.31)	21.5-30.6 (+/- 0.54)	24.7-30.1 (+/- 0.32)
PRV			30.1-37.0 (+/- 0.57)	30.3-37.0 (+/- 0.55)	20.2-37.0 (+/- 1.17)	25.1-37.0 (+/- 0.86)	17.2-25.5 (+/- 0.74)	15.0-28.6 (+/- 1.25)	20.9-26.9 (+/- 0.66)	17.3-28.8 (+/- 1.19)	21.2-25.5 (+/- 0.42)	23.5-28.3 (+/- 0.50)	23.6-29.1 (+/- 0.39)	23.8-30.8 (+/- 0.44)
Ctrl	37.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0
Day 0	37.0	37.0												

969

970 **S5 Table. PRV Ct values in heart and blood.** Ct values are presented as range (min to max)

971 and +/- SEM.

972

973
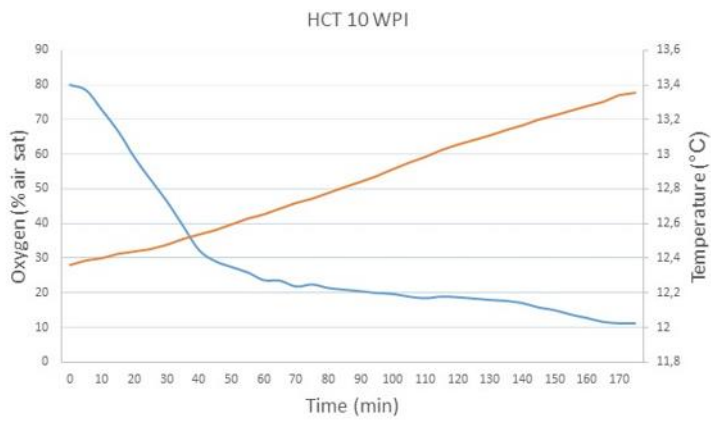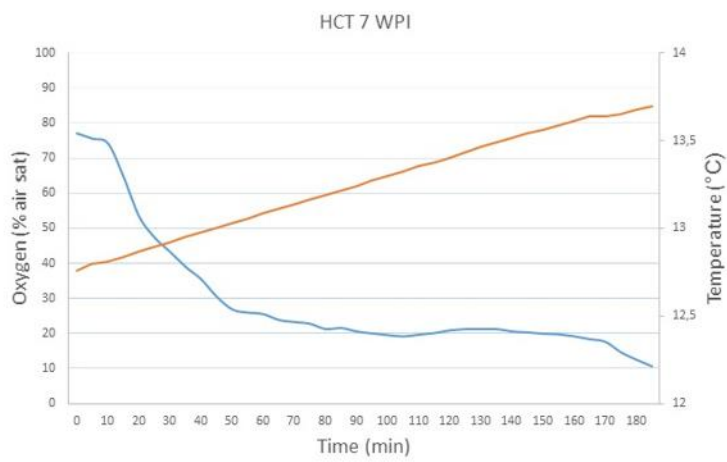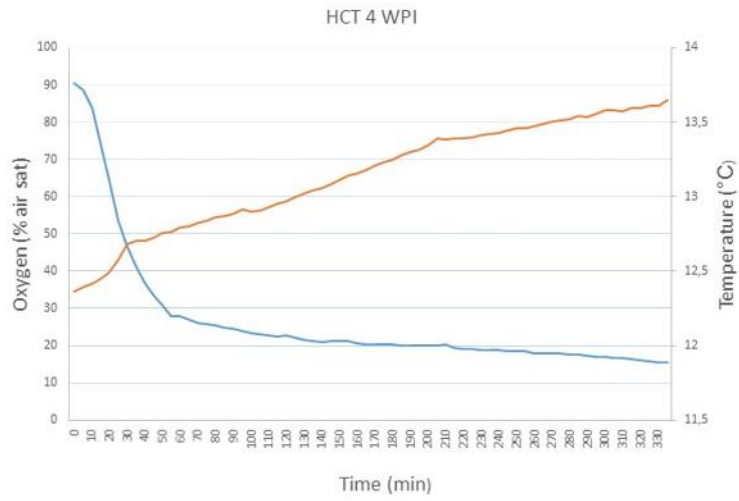
S6 Fig. Time-course of water oxygenation and temperature for each HCT.

975
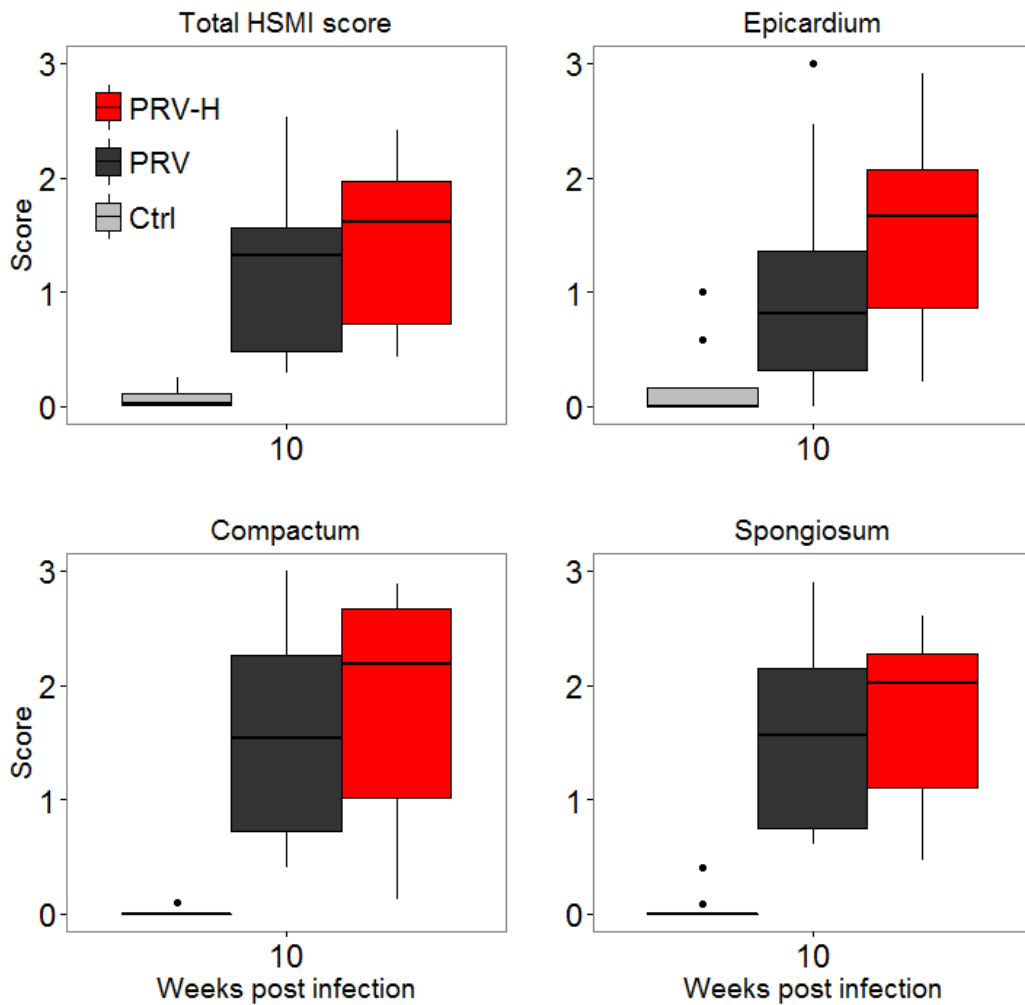
S7 Fig. Histological scoring of inflammatory changes in the heart – Heart rate.
Development of inflammatory changes is displayed for fish included in the heart rate
measurement at 10 weeks post infection (WPI). Ctrl: non-infected controls, PRV: PRV infected
fish, PRV-H: PRV infected fish exposed to periodic hypoxic stress. Inflammatory changes in
epicardium, compactum and spongiosum were scored from sections of the heart ventricle using
a continuous visual analogue scale ranging from 0-3. The total HSMI score was calculated from
the mean of scores from the separate heart compartments. The lower and upper border of boxes
indicates the 25th and 75th percentiles, respectively and the centerline indicates the 50th
percentile. The upper and lower whiskers correspond to the highest and lowest value of the
1.5*IQR (inter-quartile range).

A

B

Coefficients of the regression line modelling heart rate (BPM)
by virus load (Ct values)

<u>R out put:</u>

```
(Intercept)      temp$virus_load
106.3078418    0.8922527
```

<u>R out put:</u>

```
Pearson's product-moment correlation

data:  temp$avHR and temp$virus_load
t = 0.66219, df = 18, p-value = 0.5162
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
-0.3094230  0.5586131
sample estimates:
cor
0.1542136
```

986

987 **S8 Fig. Pearson correlation analysis between PRV Ct values and heart rate.**

988

989
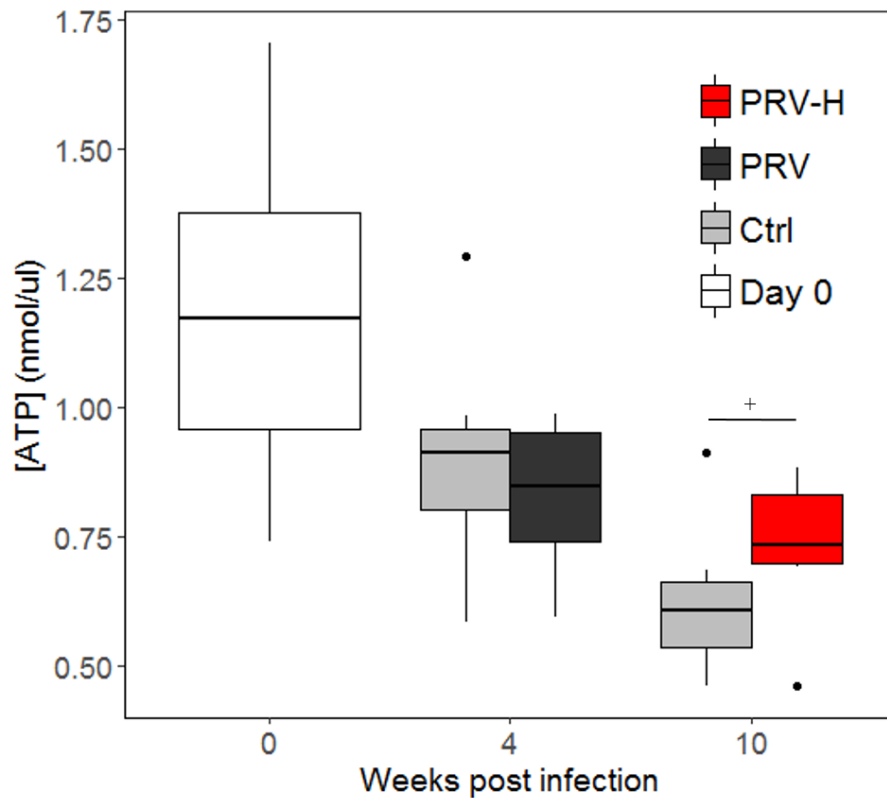
S9 Fig. ATP levels in every group at Day 0 and at 4 and 10 weeks post infection. Ctrl: non-infected controls, PRV: PRV-infected fish, PRV-H: PRV-infected fish exposed to periodic hypoxic stress. The lower and upper border of boxes indicates the 25th and 75th percentiles, respectively and the centerline indicates the 50th percentile. The upper and lower whiskers correspond to the highest and lowest value of the 1.5*IQR (inter-quartile range). Significance is indicated by + with a $p = 0.07$.

996
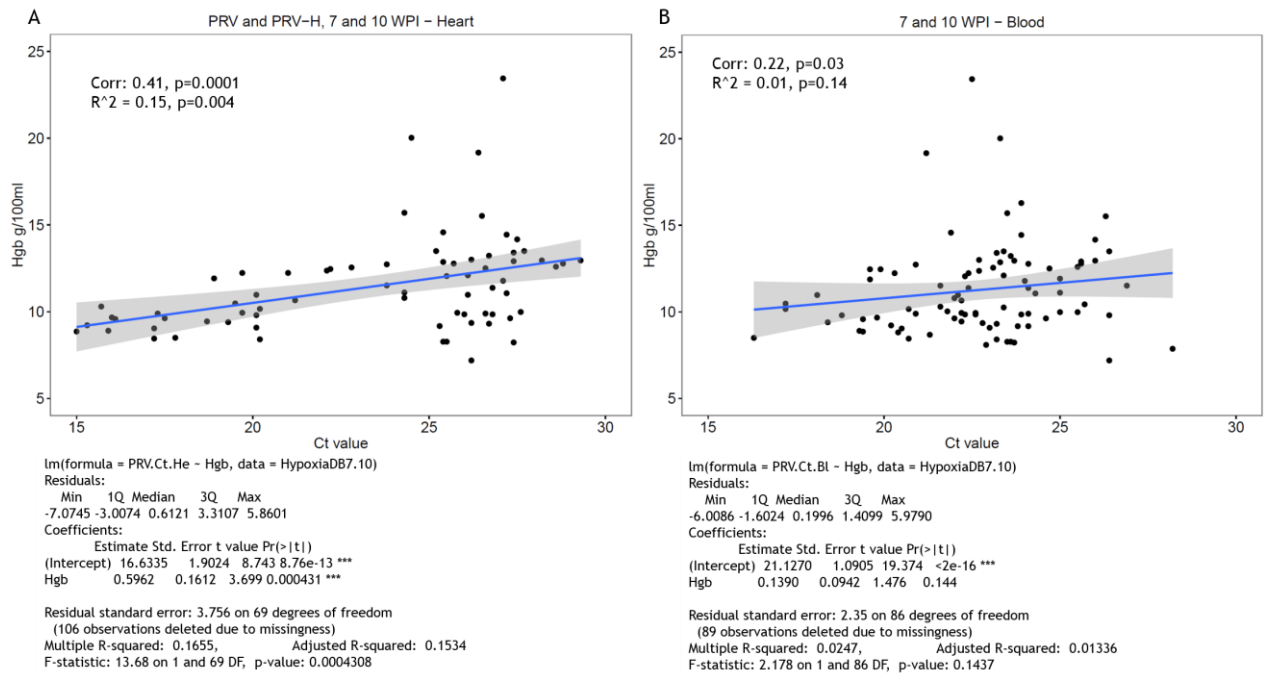
S10 Fig. Pearson correlation analysis and linear regression analysis of PRV Ct values and hemoglobin (Hb) concentrations. The analysis was performed on merged data-points from 7 and 10 weeks post infection. A. Correlation and linear regression output between PRV Ct values in heart and Hb concentrations (Hgb g/100 ml). B. Correlation and linear regression output between PRV Ct values in blood and Hb concentrations (Hgb g/100 ml).

Paper II

VR **VETERINARY RESEARCH**

RESEARCH ARTICLE

CrossMark

Experimental *Piscine orthoreovirus* infection mediates protection against pancreas disease in Atlantic salmon (*Salmo salar*)

Morten Lund[1†], Magnus Vikan Røsæg[2,3†], Aleksei Krasnov[4], Gerrit Timmerhaus[4], Ingvild Berg Nyman[3], Vidar Aspehaug[5], Espen Rimstad[3] and Maria Krudtaa Dahle[1*]

Abstract

Viral diseases are among the main challenges in farming of Atlantic salmon (*Salmo salar*). The most prevalent viral diseases in Norwegian salmon aquaculture are heart and skeletal muscle inflammation (HSMI) caused by *Piscine orthoreovirus* (PRV), and pancreas disease (PD) caused by Salmonid alphavirus (SAV). Both PRV and SAV target heart and skeletal muscles, but SAV additionally targets exocrine pancreas. PRV and SAV are often present in the same locations and co-infections occur, but the effect of this crosstalk on disease development has not been investigated. In the present experiment, the effect of a primary PRV infection on subsequent SAV infection was studied. Atlantic salmon were infected with PRV by cohabitation, followed by addition of SAV shedder fish 4 or 10 weeks after the initial PRV infection. Histopathological evaluation, monitoring of viral RNA levels and host gene expression analysis were used to assess disease development. Significant reduction of SAV RNA levels and of PD specific histopathological changes were observed in the co-infected groups compared to fish infected by SAV only. A strong correlation was found between histopathological development and expression of disease related genes in heart. In conclusion, experimentally PRV infected salmon are less susceptible to secondary SAV infection and development of PD.

Introduction

Virus infections are a continuous challenge in large-scale aquaculture of Atlantic salmon (*Salmo salar*). Environmental factors, high intensity production and infectious agents affect both welfare and production [1–3]. The two most prevalent viral diseases in Norwegian Atlantic salmon aquaculture are heart and skeletal muscle inflammation (HSMI) and pancreas disease (PD) [4]. *Piscine orthoreovirus* (PRV) is associated with HSMI, is ubiquitous in sea reared Atlantic salmon in Norway and often detected without any signs of disease [5, 6]. Pancreas disease is caused by Salmon pancreas disease virus, more commonly known as Salmonid alphavirus (SAV). The two viral diseases have overlapping geographic distributions

[4, 7], both target heart and skeletal muscle and may co-infect Atlantic salmon [8–10].

PRV is a non-enveloped virus with a segmented, double stranded RNA genome, belonging to the genus *Orthoreovirus* in the family *Reoviridae* [5, 11]. Salmonid erythrocytes are major target cells for PRV and more than 50% of these cells may be infected in the peak phase of the infection [12]. In later stages of the infection, PRV infects myocytes of the heart and skeletal muscles [13]. The histopathological changes in heart and skeletal muscle gave the condition its name in the late 1990s, and later the association with PRV was established [5, 14].

SAV is an enveloped virus with a single-stranded positive sense RNA genome of the family *Togaviridae* [15]. Pancreas, heart and skeletal muscle are the main target tissues. The disease is recognized by growth retardation, reduced slaughter quality and increased mortality [9, 16, 17]. Histopathological changes are characterized by acute necrosis of exocrine pancreas, myocardial and skeletal muscle necrosis with subsequent inflammation [9]. The

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pancreatic lesions are a hallmark of PD and hence used for diagnostic differentiation from HSMI and cardiomyopathy syndrome (CMS) [9]. However, PRV and SAV have common target tissues in heart and skeletal muscles, making interactions between the two viral infections possible.

SAV is divided into six different phylogenetic subtypes [18] and subtypes 2 and 3 are present in Norwegian aquaculture [19, 20]. The two subtypes show approximately 7% differences in nucleotide sequence [19], are endemically present in separate geographic areas and differ in virulence [20–22]. The mechanisms behind the difference in virulence are unknown. No stereotypical difference is described between subtype 2 and 3 [23]. PD outbreaks vary in duration, severity and accumulated mortality [24], indicating that factors other than SAV influences disease development. Interaction with other infectious agents may be such a factor.

Protection to a secondary virus infection induced by an unrelated primary virus infection has been recognized since the 1950s [25], and has also been described for several viruses infecting salmonid fish [26–30]. However, the duration of the protection of rainbow trout to infectious hematopoietic necrosis after primary infection with the non-virulent cutthroat trout virus was found to be no more than 4 weeks [26]. In addition, some viral infections in terrestrial animals are shown to aggravate disease development of a secondary viral infection [31, 32].

The purpose of this study was to determine if a primary PRV infection alters the outcome of a subsequent SAV infection. Experimental infection trials were performed to compare disease development, viral kinetics and expression of disease associated genes between PRV-SAV co-infected and SAV infected fish.

Materials and methods
Fish
Sea water adapted Atlantic salmon ($N = 987$) of a SalmoBreed strain (Bergen, Norway) were used in the study (VESO Vikan, Namsos, Norway). The post smolts were transferred to sea water two weeks before PRV challenge. Prior to challenge, the fish were screened and found to be negative for PRV, infectious pancreatic necrosis virus (IPNV) and SAV by reverse transcriptase (RT) qPCR. PRV shedders ($N = 5$) sampled four weeks after PRV challenge were confirmed negative for Atlantic salmon calicivirus [33]. During the challenge trial the fish were kept in filtered and UV-radiated seawater (34 ‰ salinity), 12 °C (± 1 °C) and on 24 h light. The fish were fed 1% of total biomass per day and starved for 24 h prior to handling and sampling. Before sampling, the fish were euthanized by bath immersion containing benzocaine chloride (1 g/5L water) (Apotekproduksjon AS, Oslo,

Norway) for 5 min. The challenge trial was approved by the Norwegian Animal Research Authority and performed in accordance with the recommendations of the current animal welfare regulations: FOR-1996-01-15-23 (Norway).

Experimental challenge
PRV challenge
The inoculum (consisting of pelleted blood cells) was collected in a previous cohabitation trial (VESO Vikan), i.e. the second passage in experimental fish, and originated from a Norwegian field outbreak of HSMI in 2012. RTqPCR was performed as earlier described [34] and a high level of PRV RNA was indicated (Ct 17.3, using a total RNA input of 100 ng in the RTqPCR). The blood pellet was dissolved 1:1 in PBS and stored at -80 °C. On Day 0 of the trial, the blood pellet and PBS solution was thawed on ice, diluted 1:2 in PBS and 0.1 mL of the inoculum was i.p. injected into the anesthetized shedders. The inoculum was confirmed negative for IPNV, infectious salmon anemia virus (ISAV), SAV, piscine myocarditis virus (PMCV) by RTqPCR. After i.p. inoculation, the shedders ($N = 363$) were marked by adipose fin removal and placed in a tank containing naïve fish ($N = 396$). Four weeks post PRV shedder introduction (WPC-PRV), the PRV shedders were removed and the cohabitants were distributed into four tanks. As displayed in Figure 1, at 4 WPC-PRV, two tanks containing PRV cohabitants ($N = 80$) were supplied with either SAV2 ($N = 20$) or SAV3 ($N = 20$) shedders (1:4 ratio, shedder:cohabitant) starting the early co-infection. While the two other tanks contained PRV cohabitants until 10 WPC-PRV (indicated by "PRV" in Figure 1) and were sampled as PRV controls at 7 and 10 WPC-PRV. At 10 WPC-PRV, the PRV cohabitants ($N = 80$) in the tanks, were supplied with SAV2 ($N = 20$) or SAV3 ($N = 20$) shedders, initiating the late co-infection. Hence, cohabitants in the early and late co-infection were challenged with SAV shedders 6 and 12 weeks post sea water transfer, respectively.

SAV challenge
Naïve fish ($N = 218$) were kept in a separate tank ("Naïve fish 2", Figure 1). SAV2 or SAV3 shedders were i.p. injected with 0.1 mL of cell culture medium containing SAV2 or SAV3 at a concentration of 10^4 TCID$_{50}$/mL. The SAV inocula were prepared as described earlier [21]. SAV shedders were marked by maxilla cutting. After injection, the SAV shedders were kept in separate tanks for four days before being introduced to the cohabitants. SAV2 or SAV3 shedders ($N = 13$) were placed in tanks with respective SAV subtype control tanks ($N = 52$ in each tank) (Figure 1). Naïve fish to be i.p. injected with

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Figure 1 The co-infection challenge trial. The timeline above indicates weeks post PRV shedder introduction (WPC-PRV). Blue down arrow indicates transfer to sea water (SW). Yellow down arrow and yellow up arrow indicates introduction and removal, respectively, of PRV shedder fish. Red down arrow indicates introduction of SAV shedders. "Naïve fish 1" indicate the experimental fish before virus challenge, "Naïve fish 2" indicate fish dedicated to be SAV shedders and SAV controls at 4 WPC-PRV and "Naïve fish 3" indicate fish dedicated to be SAV shedders at 10 WPC-PRV. Blue box named "PRV cohabitation" indicates cohabitant fish exposed to 50% PRV shedders from 0 to 4 WPC-PRV. Blue box named "PRV" indicates PRV cohabitants from 4 to 10 WPC-PRV without PRV shedders. Boxes indicating a 6 week period of exposure to 20% SAV shedders are colored red (SAV only) and green (PRV-SAV). Co-infection induced at 4 WPC-PRV and 10 WPC-PRV is denoted PRV-SAV-early and PRV-SAV-late, respectively.

SAV at 10 WPC-PRV were kept in a separate tank from 4 to 10 WPC-PRV ("Naïve fish 3" in Figure 1). Time after introduction of SAV shedders will be referred to as weeks post SAV shedder introduction (WPC-SAV). Organ samples from heart on RNA*later*™ (Ambion Inc., USA) and heparinized blood were collected from naïve fish ($N = 4$) before SAV challenge. These were confirmed negative for SAV and PRV by RTqPCR. Due to differences in virulence and geographic distribution [21, 22], both SAV2 and SAV3 were included in the study.

Sampling
Eight cohabitants were sampled from the PRV only and PRV-SAV co-infected groups, whereas six cohabitants were sampled from the SAV control groups at each sampling. Eight fish sampled prior to PRV challenge served as negative controls. Weight and fork length was registered for all sampled cohabitants and Fulton's condition factor (k-factor) was calculated (k-factor = weight in grams/ length in cm^3 × 100).

Tissue samples for histopathological evaluation (heart, pyloric caeca including exocrine pancreas and red and white skeletal muscle including the lateral line) were collected and fixed in 10% phosphate buffered formalin. After 24 h, the formalin was replaced with 70% ethanol and stored at 4 °C until further handling.

Two pieces of 2 mm^3 from heart were collected on prefilled 1.0 mL tubes (FluidX® Ltd, UK) with 0.5 mL RNA*later*™ for RTqPCR analysis. Heparinized blood was collected from the caudal vein.

Histopathology and immunohistochemistry
Samples for histopathology were processed and stained with hematoxylin and eosin following standard procedures. The sections from heart, pyloric caeca and skeletal muscle were examined blind and scored for pathological changes in an ordinal system (0, 1, 2, and 3), based on Taksdal et al. and McLoughlin et al. [21, 35]. The scoring performed in this study was modified and extended to include a separate score for acute myocardial necrosis and epicarditis as the former is a hallmark in PD development and the latter is observed in both PD and HSMI [9]. The scoring criteria for exocrine pancreas, myocardial degeneration and inflammation, acute myocardial necrosis, epicarditis and inflammation in skeletal muscle are displayed in Additional file 1.

Immunohistochemistry for detection of PRV and SAV in heart tissue were performed as described earlier for detection of PRV [13]; polyclonal rabbit anti-σ1 serum (1:2500) [13] for PRV and monoclonal murine anti-E2 (17H23) (1:2000) [36] for SAV were used as primary antibodies. Both were incubated overnight in a humidity chamber, SAV at room temperature and PRV at 4 °C. Biotinylated goat anti-rabbit (1:200) and biotinylated rabbit anti-mouse (1:300) were used as secondary antibodies (Dako, Agilent Technologies, Glostrop, Denmark). Vectastain ABC-AP kit (Vector, Laboratories, Burlingame, CA, USA) was used for visualization. Heart tissues from double infected fish tissues were investigated. As negative and positive controls, slides with SAV or PRV single infected tissues were included.

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RNA isolation and RTqPCR

All samples, including heparinized blood, were shipped cool (5–10 °C) and arrived within 24 h to the Norwegian Veterinary Institute laboratory after sampling. Tissue samples on RNA*later*™ were placed at −20 °C until further analysis. A sub-sample of 200 μL from each heparinized blood sample was subsequently shipped cold, together with heart samples on RNA*later*™, to PatoGen AS for virus analysis.

PatoGen AS performed RNA extraction and RTqPCR analysis for PRV and SAV transcripts in heart and heparinized blood. The RTqPCR assay targeting PRV is validated to ISO17025 standards and was described by Glover et al. [37]. The SAV assay is validated and accredited to ISO17025 standards and was performed as described earlier [38]. Samples were defined as positive when having a PRV or SAV Ct lower than 37.0. Elongation factor 1α (EF1α) served as an internal reference gene [39] for all RTqPCR assays performed. PRV and SAV Ct values were normalized to EF1α Ct values ($\Delta Ct = Ct^{target} - Ct^{EF1\alpha}$). After finalizing the virus analyses, RNA (in RNase free H_2O) extracted from heart and blood by PatoGen AS was shipped frozen on dry-ice, overnight to the NVI. RNA quantification and purity was determined using NanoDrop 2000 UV–Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Finally, 1 μL RNase Out (0.5 U/μL, Life technologies) was added and the RNA was stored at −80 °C until gene expression analysis.

For gene expression analysis, cDNA was produced from 600 ng total RNA using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Quantitative PCR was performed using 15 ng (5 μL of 3 ng/μL) cDNA input per reaction using Maxima SYBR Green (Thermo Scientific) with 10 μM of both primers. Primers are listed in Table 1. The cycling conditions used were 95 °C for 10 min, then 40 cycles of 95 °C/15 s, 60 °C/30 s and 72 °C/30 s in a Mx3005P (Stratagene, La Jolla, CA, USA). A seven-point concentration grade standard curve (40–0.675 ng) was run during testing of the primer pairs.

Microarray analyses

The analyses were carried out using NOFIMA's Atlantic salmon oligonucleotide microarray SIQ-6 and bioinformatic package STARS [40]. The platform includes 15 k unique probes to protein encoding transcripts; the genes were annotated by functions (GO), pathways (KEGG) and custom vocabulary. Microarrays were manufactured by Agilent Technologies (Santa Clara, CA, USA) and unless indicated otherwise, the reagents and equipment were purchased from the same source. The microarray analyses were performed on RNA from heart tissue that was shipped overnight from NVI to NOFIMA on dry ice. RNA from uninfected hearts, sampled at Day 0, was used as a common reference in all hybridizations. RNA amplification and labelling were performed with a Two-Color Quick Amp Labelling Kit and a Gene Expression Hybridization kit was used for fragmentation of labelled RNA. Total RNA input for each reaction was 500 ng. After overnight hybridization in an oven (17 h, 65 °C, rotation speed 0.01 g), arrays were washed with Gene Expression Wash Buffers 1 and 2 and scanned with a GenePix 4100A (Molecular Devices, Sunnyvale, CA, USA). GenePix Pro 6.0 was used for spot to grid alignment, assessment of spot quality, feature extraction and quantification. Subsequent data analyses were performed with STARS. After filtration of low quality spots flagged by GenePix, Lowess normalization of $\log_2$-expression ratios (ER) was

Table 1 Primers used for gene expression analyses

Target name	Sequence	Amplicon length	Genbank no.
Calsequestrin	Fwd: ATCCAGATGACTTCCCGCTG Rev: CTGGGGAGAGCCTAGGTCAAT	72	NM_001141681.1
Matrix metalloproteinase 13	Fwd: AGTGTCCAGCACAAATGACCT Rev: CTCAACTGCTGATCCACTGGT	78	XM_014163130.1
Interleukin 1-receptor accessory protein-like 2	Fwd:CTGGCTGGTCAATGGGACAT Rev: GTGGACCTGAAGTCCTCTGC	144	XM_014137694.1
Neuropeptide Y1	Fwd: GCTACCCGGTCAAACCTGAA Rev: GGACTGTGGGAGCGTATCTG	194	XM_014178359.1
Serum amyloid A5 protein	Fwd: GGTGCTAAAGACATGTGGCG Rev: CCACTGGAACCCTGAACCAT	173	NM_001146565.1
Arginase 1	Fwd: TGGCGATGTGCCTTTGATTT Rev: ATCCCGCGGTTGTCCTTTT	208	NM_001141316.2
Arginase 2—mitochondrial	Fwd: AACACAGGGTTGTTGTCGGT Rev: AGAGTCGAAGCTGTTCCGTG	193	XM_014211724.1

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performed. Genes that passed the quality control in more than half of the samples were included in the subsequent analyses. Differential expression was assessed by criteria: ER > 1.75-fold and $p < 0.05$.

Data analysis and statistics

The statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software inc., USA). Differences in viral RNA levels were calculated based on ΔCt values using the non-parametric Mann–Whitney unpaired rank test. Differences in gene transcript levels in heart tissue and histopathological scores between the groups were examined using the non-parametric Mann–Whitney unpaired rank test. An unpaired student t test was used to examine differences in k-factor and weight. Spearman's rank correlation was calculated using STATA 13.1 (StataCorp, USA), for associations between viral RNA levels in SAV cohabitants and histopathology score of acute myocardial necrosis. In addition, association between gene expression (ΔCt) and histopathology score of both myocardial degeneration and inflammation and acute myocardial necrosis was calculated. In all calculations of differences, a $p < 0.05$ was considered statistically significant.

Results

The cohabitant challenge experiment is displayed in Figure 1. Weeks post PRV shedder introduction are abbreviated as WPC-PRV and weeks post SAV shedder introduction as WPC-SAV. The challenge trial consisted of 4 or 10 weeks of PRV infection alone (WPC-PRV) and a subsequent 6-week PRV-SAV co-infection period. The different time-points for SAV shedder introduction, i.e. 4 and 10 WPC-PRV, are termed early and late co-infection, respectively. Hence, the terms used are PRV-SAV-early or PRV-SAV-late. The SAV control fish were challenged simultaneously with the early co-infection. The results originate from cohabitant fish unless specified otherwise.

Mortality and growth

Mortality was low during the experiment. Three (0.76%) PRV infected fish died during the first 4 weeks, while one fish died in the PRV-SAV2-early group (1.25%) between 4 and 10 WPC-PRV. The accumulated mortality at 6 WPC-SAV was 2.0 and 5.7% in the SAV2 and SAV3 controls, respectively. In the late co-infection there were no mortalities.

On Day 0, the mean weight, length and condition (k)-factor was 105.6 g (range 70.6–160.4 g), 21.2 cm (range 18.5–24.5 cm) and 1.09 (range 1.02–1.17), respectively. At the time of SAV shedder introduction, the mean weight at 4 WPC-PRV was 114.7 g ($N = 16$, PRV early group) and 109.3 g ($N = 4$, naïve fish) and at 10 WPC-PRV, the

mean weight was 157.4 g ($N = 16$, PRV late group). At 10 WPC-PRV, all groups had increased average weight and length. However, the mean k-factor was reduced in the PRV-SAV2-early (1.06), SAV2 (1.02) and SAV3 (1.02) groups and increased in PRV controls (1.10) and the PRV-SAV3-early group (1.11). The difference between the SAV control groups compared to the PRV controls and the PRV-SAV3-early group were significant ($p < 0.05$) at 10 WPC-PRV. At the end of the trial, i.e. 16 WPC-PRV, the PRV-SAV2-late and PRV-SAV3-late groups had a mean k-factor of 1.17 and 1.11, respectively. Additional file 2 shows detailed range of weight, length and k-factor.

PRV infection kinetics

At day 0, the fish were confirmed negative for both PRV and SAV by RTqPCR. Successful transmission and infection of the cohabitants with PRV were confirmed by detection of viral RNA in blood and heart (Figures 2A–D). PRV was first detected in cohabitant fish at 3 WPC-PRV and the level of PRV RNA peaked at 5 WPC-PRV in blood (mean Ct 14.2) and 6 WPC-PRV in heart (mean Ct 18.3). High PRV RNA levels were present in the fish until the end of the experiment at 16 WPC-PRV (Figures 2C and D).

Histopathological changes including epicarditis and myocardial degeneration and inflammation in red skeletal muscle were in accordance to experimentally induced HSMI (Additional file 3), as previously described [13]. There were no differences in ΔCt values of PRV RNA or histopathological changes between the PRV controls and PRV-SAV groups. Likewise, there were no differences between the co-infected groups when comparing ΔCt values of PRV transcripts except a significantly lower ΔCt value in PRV-SAV3-late group compared to PRV-SAV2-late group at 12 WPC-PRV, $p < 0.05$ (Figures 2C and D).

SAV infection kinetics

SAV2 and SAV3 shedders were confirmed SAV positive by RTqPCR in heart and blood. The histopathological changes were characteristic for PD, i.e. loss of exocrine pancreatic tissue, acute myocardial necrosis and myocarditis.

SAV challenge at 4 WPC-PRV (early co-infection)

In the PRV-SAV2-early group, the levels of SAV RNA were significantly lower in blood at 3, 4 and 6 WPC-SAV compared to the SAV2 controls ($p < 0.05$) and in heart at 4 and 6 WPC-SAV ($p < 0.05$). At both time points, 3/8 fish were SAV negative in heart in the co-infected group.

The SAV RNA levels in the SAV3 control group peaked 3 WPC-SAV, however the PRV-SAV3-early group did not reach the same level (Figures 3C and D). At 3 and 4 WPC-SAV, the SAV RNA levels in blood was undetectable

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Figure 2 PRV RNA kinetics. PRV ΔCt values (PRV Ct–EF1α Ct) in blood and heart from individual co-challenged fish (black and grey dots) and PRV control fish (red x) assayed by RTqPCR targeting PRV RNA segments. Red arrow indicates introduction of SAV shedders early (**A**, **B**) or late (**C**, **D**). Weeks post PRV shedder introduction (WPC-PRV) are indicated at the x-axis. N positive individuals (raw Ct <37.0) of total sampled fish is tabulated under each sub-figure. Asterisk indicates significant (*p* < 0.05) differences in PRV RNA levels.

in more than 50% of the fish in the co-infected group and significantly lower than in the SAV3 control group (*p* < 0.05). The SAV RNA level in heart was significantly lower in the co-infected fish at 4 and 6 WPC-SAV compared to the SAV3 controls (*p* < 0.05).

SAV challenge at 10 WPC-PRV (late co-infection)

In the late co-infection, i.e. addition of SAV shedders at 10 WPC-PRV, SAV RNA was first detected in the PRV-SAV2-late group in blood and heart at 3 WPC-SAV and peaked at week 4 post SAV introduction, which was one week later than the control group (Figures 4A and B). The SAV RNA level in blood was significantly lower in the co-infected group at 3 WPC-SAV compared to the SAV control groups (*p* < 0.05). Furthermore, the co-infected

group had a significantly lower SAV RNA level in heart compared to the controls at 3 WPC-SAV (*p* < 0.05).

In the PRV-SAV3-late group the SAV RNA levels did not show the same delay as observed for the PRV-SAV2-late group (Figures 4C and D). SAV RNA was first detected 2 WPC-SAV and reached peak levels 4 WPC-SAV in both heart and blood. However, at 3 and 4 WPC-SAV the SAV RNA level was significantly lower in heart in the co-infected group (*p* < 0.05). In blood, the SAV RNA level was significantly lower at 3 WPC-SAV in the PRV-SAV3-late group compared to the SAV3 controls (*p* < 0.05).

During the co-infection, the SAV shedders also got infected with PRV (Figure 5). Although not significant, the PRV RNA levels in the SAV2 shedders after 6 WPC-SAV, were higher in heart when compared to SAV3 shedders.

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Figure 3 SAV RNA kinetics, early co-infection. SAV ΔCt values (SAV Ct–EF1α Ct) in blood and heart from individual co-challenged fish (black dots) and SAV control fish (red x) assayed by RTqPCR targeting SAV RNA segments. Red arrow indicates introduction of SAV2 shedders (**A**, **B**) or SAV3 shedders (**C**, **D**). Weeks post SAV shedder introduction (WPC-SAV) are indicated at the x-axis. N positive individuals (raw Ct < 37.0) of total sampled fish is tabulated under each sub-figure. Asterisk indicates significant ($p < 0.05$) differences between co-challenged groups and SAV control group.
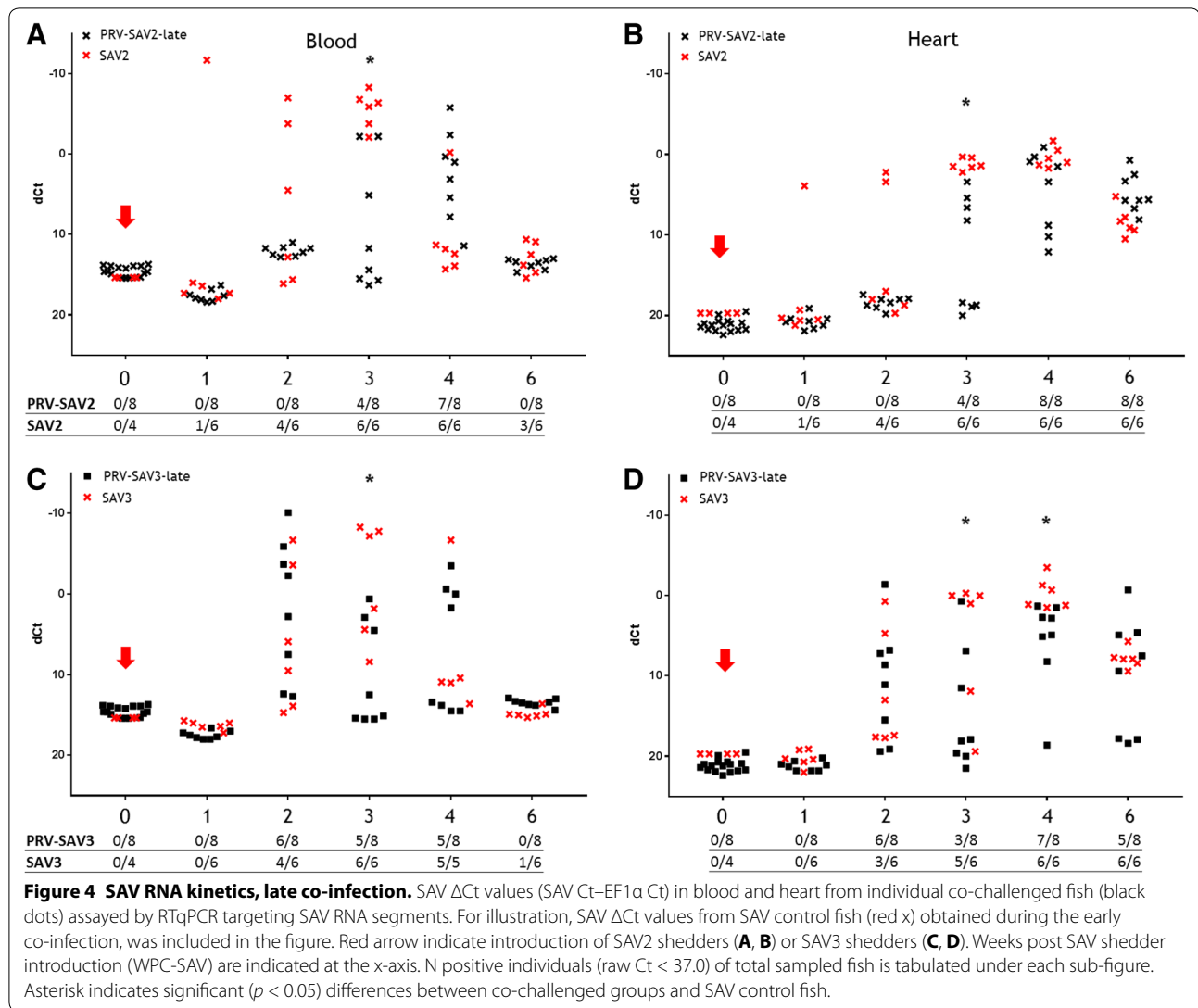
Histopathology in co-infected groups

Histopathological scoring of changes in pancreas and acute myocardial necrosis showed a reduction in the co-infected groups compared with SAV control groups (Figures 6 and 7). Using a non-parametric rank test of the ordinal histopathological score, changes in pancreas were found to be significantly lower at 4 and 6 WPC-SAV in both early and late co-infection compared to the SAV control groups ($p < 0.05$), except at 4 WPC-SAV in the PRV-SAV3-late group (Figures 6 and 7). The PRV-SAV2-late group had also a significantly lower score in pancreas compared to the SAV2 control group at 3 WPC-SAV ($p < 0.05$). The prevalence of acute myocardial necrosis was significantly reduced at 4 WPC-SAV in the co-infected groups compared to the SAV controls ($p < 0.05$). At 6 WPC-SAV, the prevalence of acute myocardial necrosis was significantly lower in the PRV-SAV3-late and PRV-SAV2-early groups compared to the SAV controls ($p < 0.05$).

The SAV RNA levels (ΔCt) and histopathology score of acute myocardial necrosis showed a strong positive Spearman's rank correlation ($r_s = 0.81$) in the SAV3 control group, $p < 0.05$ ($N = 28$), whereas a weaker correlation ($r_s = 0.59$) was seen in the SAV2 controls, $p < 0.05$ ($N = 28$).

Immunohistochemistry (IHC) was performed on sections of heart tissue from single infected and co-infected fish. The fish were selected based on high viral levels (indicated by low Ct levels) of both viruses. The fish presented in Figure 8 were sampled 7 WPC-PRV, which for the SAV controls and co-infected groups correspond to three weeks post SAV introduction (3 WPC-SAV) in the early co-infection. Virus Ct values in the respective heart tissues are noted in Figure 8. In the SAV3 infected fish, sparse but distinct staining restricted to single cells was observed using SAV antibodies. The PRV antibodies yield a weak pink background color in both PRV infected and SAV infected heart tissues. However, distinct staining

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Figure 4 SAV RNA kinetics, late co-infection. SAV ΔCt values (SAV Ct–EF1α Ct) in blood and heart from individual co-challenged fish (black dots) assayed by RTqPCR targeting SAV RNA segments. For illustration, SAV ΔCt values from SAV control fish (red x) obtained during the early co-infection, was included in the figure. Red arrow indicate introduction of SAV2 shedders (**A**, **B**) or SAV3 shedders (**C**, **D**). Weeks post SAV shedder introduction (WPC-SAV) are indicated at the x-axis. N positive individuals (raw Ct < 37.0) of total sampled fish is tabulated under each sub-figure. Asterisk indicates significant (*p* < 0.05) differences between co-challenged groups and SAV control fish.
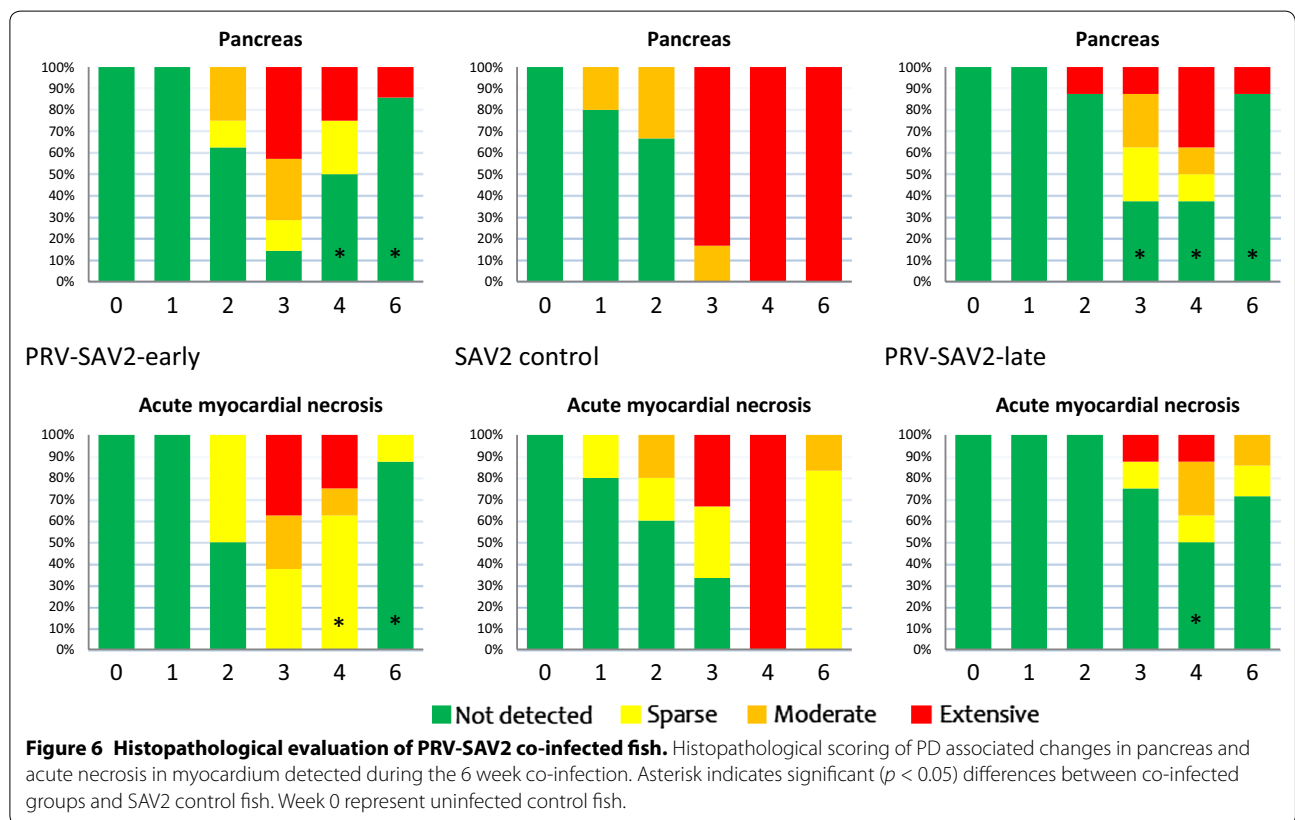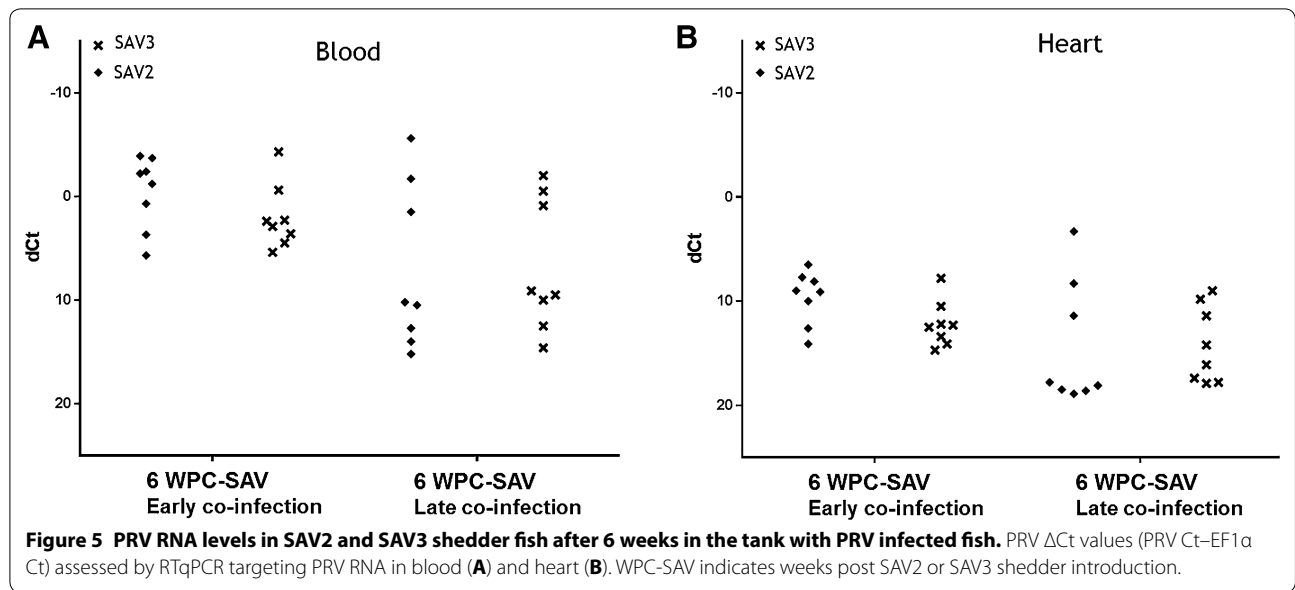
was only observed in PRV infected heart. Staining of both SAV and PRV antibodies were achieved in two separate sections of a co-infected heart. In the co-infected heart tissue more diffuse staining was observed in epicard, interpreted as unspecific binding for both SAV and PRV antibodies. When staining the control tissue with PRV antibodies, a weak pink background color was observed. Additional files 4, 5 and 6 include more detailed pictures. The IHC demonstrates the presence of PRV and SAV in the same areas of a co-infected heart section. However, no individual cells could be defined as co-infected.

PRV-SAV co-infection and gene expression linked to heart pathology
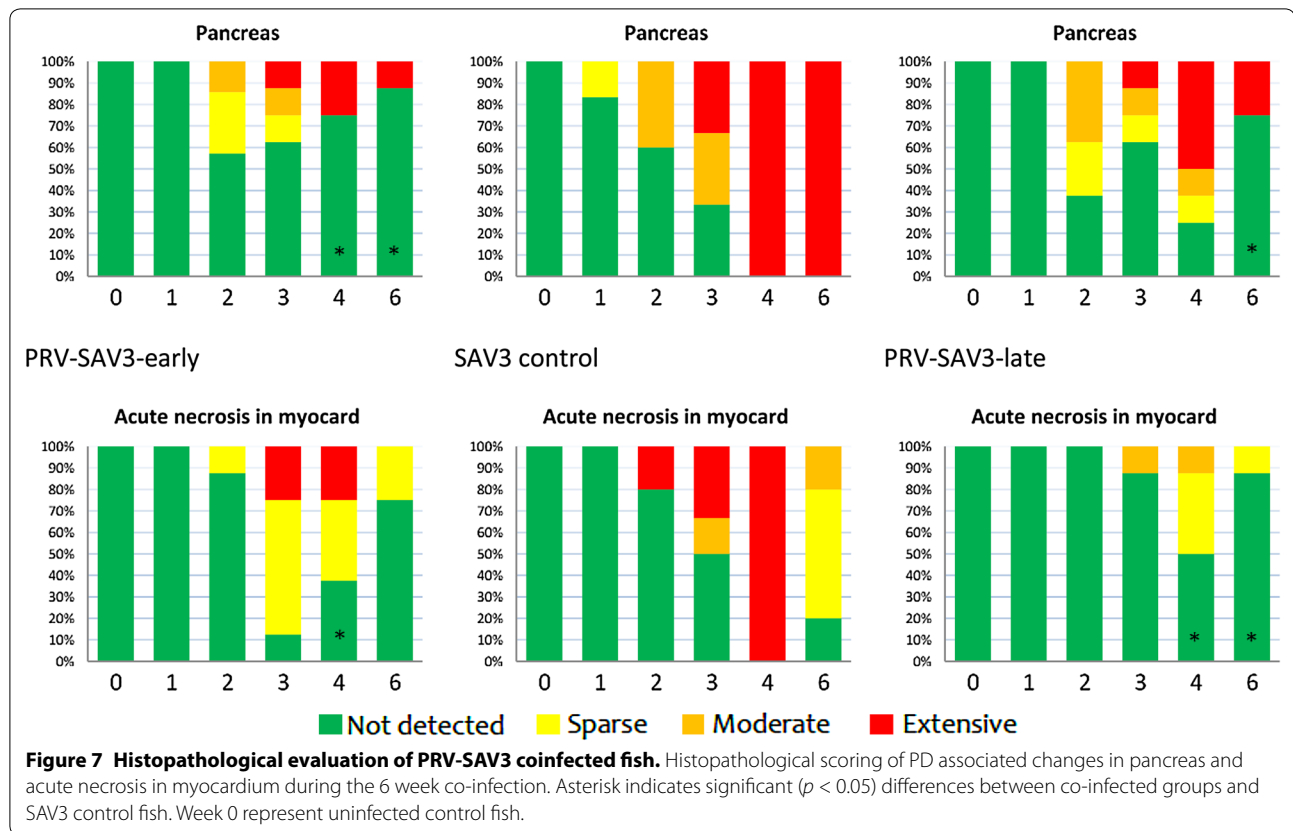
Microarray analysis was performed on hearts sampled at 4 and 6 WPC-SAV from the late co-infection and differences between the SAV3 control and PRV-SAV3-late

group were analyzed (Table 2). Genes were selected based on their correlation with severity of pathological changes in heart induced by SAV infection, as previously reported [41]. To confirm the array results, RT-qPCR assays were run for seven selected genes, of which six showed significant differences (Figure 9). The gene regulation relative to 4 WPC-PRV (set to zero) is shown in Figure 9. At this time point the heart appeared healthy by histopathological evaluation. Gene expression at 4 and 6 WPC-SAV in both the early and the late co-infected group were compared to 4 and 6 WPC-SAV in the SAV3 control group. Significant differences were found between PRV-SAV3-late and SAV3 control for calsequestrin, neuropeptide Y-1 and interleukin 1-receptor accessory protein-like 2 (IL1R-2) at both 4 and 6 WPC-SAV, at 4 WPC-SAV for mitochondrial arginase-2 and at 6 WPC-SAV for arginase-1 and serum amyloid A5-protein (SAA5) (*p* < 0.05).

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Figure 5 PRV RNA levels in SAV2 and SAV3 shedder fish after 6 weeks in the tank with PRV infected fish. PRV ΔCt values (PRV Ct–EF1α Ct) assessed by RTqPCR targeting PRV RNA in blood (**A**) and heart (**B**). WPC-SAV indicates weeks post SAV2 or SAV3 shedder introduction.

Figure 6 Histopathological evaluation of PRV-SAV2 co-infected fish. Histopathological scoring of PD associated changes in pancreas and acute necrosis in myocardium detected during the 6 week co-infection. Asterisk indicates significant (*p* < 0.05) differences between co-infected groups and SAV2 control fish. Week 0 represent uninfected control fish.

The early co-infected groups differed significantly from the SAV controls for IL1R-2, mitochondrial arginase-2 and SAA5 at 4 WPC-SAV, and for calsequestrin, IL1R-2, arginase-1 and SAA5 at 6 WPC-SAV (*p* < 0.05). No

significant differences between the groups were found for matrix metalloproteinase 13 (MMP13) expression.

Histopathology scores (ordinal variable 0, 1, 2 and 3) of myocardial degeneration and inflammation and acute

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Figure 7 Histopathological evaluation of PRV-SAV3 coinfected fish. Histopathological scoring of PD associated changes in pancreas and acute necrosis in myocardium during the 6 week co-infection. Asterisk indicates significant ($p < 0.05$) differences between co-infected groups and SAV3 control fish. Week 0 represent uninfected control fish.

myocardial necrosis were correlated with gene expression levels from the qPCR, using Spearman's rank correlation (Table 3). The groups investigated ($N = 6$/group) were PRV controls (4 and 10 WPC-PRV), PRV-SAV3-early and -late (4 and 6 WPC-SAV) and SAV3 controls (4 and 6 WPC-SAV), making a total of $N = 46$ fish evaluated. Significant correlation was found for all gene expression levels toward the score of acute necrosis and inflammation in myocardial tissue, except for MMP13 against inflammation (Table 3). When corrected for multiple comparisons by Bonferroni-adjusted significant level, IL1R2, neuropeptide Y-1, SAA5, mitochondrial arginase-2 against necrosis and calsequestrin, IL1R-2, neuropeptide Y-1, arginase-1 against inflammation remained significant.
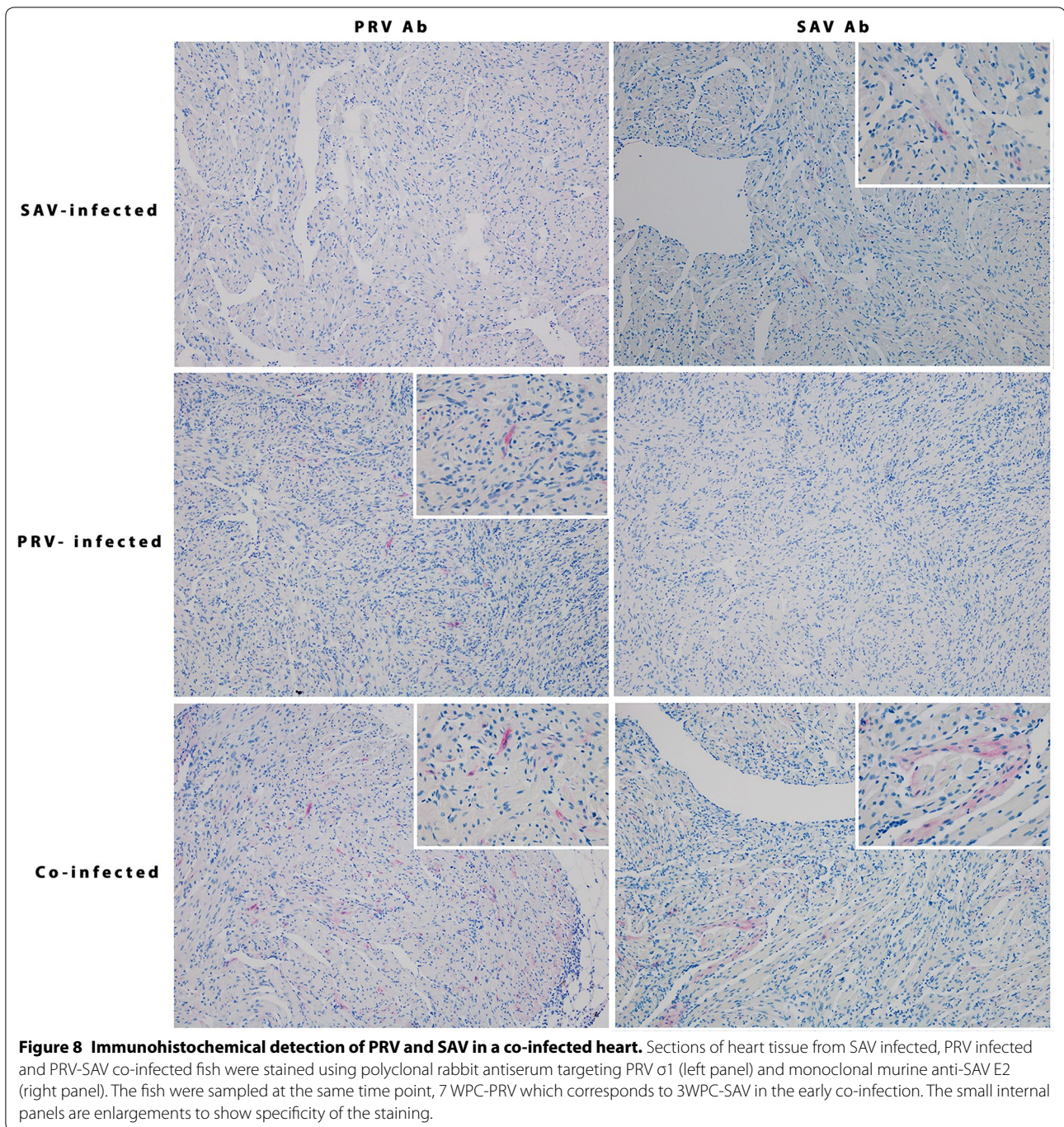
Discussion

This study demonstrates that a primary PRV infection reduces disease development of a subsequent SAV infection with either SAV2 or SAV3, as evidenced by lower levels of SAV RNA, less severe PD pathological lesions and higher condition factors in the co-infected groups. The lack of parallel groups must be accounted for when evaluating the presented results. Nevertheless, the observation of a similar reduction in disease development for both SAV subtypes in co-infected fish strengthens the validity of the results.

The most pronounced evidence of protection was a reduction in PD specific pathological lesions in exocrine pancreas. Lesions in the pancreas are a hallmark of SAV infection and used diagnostically for separation of PD and HSMI [9]. The heart is a target organ for both PRV and SAV, with myocarditis and epicarditis observed in both diseases [8, 9, 13]. This may possibly mask the protective effect on SAV induced myocarditis and epicarditis in this study. Cardiomyocytic necrosis is a typical pathological finding of early stages of SAV infection [9], and is not considered a specific feature of HSMI. In our study we found that the early co-infected groups had a significantly lower degree of acute myocardial necrosis compared to SAV controls. A recent study indicate a possible difference in susceptibility to SAV infection depending on time following sea water transfer [42]. Therefore, we cannot exclude that the size of the fish and time after sea transfer may have an impact on the difference in protection observed between the SAV only control groups and the late co-infected groups in our study.

Co-detection of PRV and SAV in hearts have been shown by RTqPCR in farmed Atlantic salmon escapees in Norway [10]. Here, we demonstrate the presence of both viruses in

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Figure 8 Immunohistochemical detection of PRV and SAV in a co-infected heart. Sections of heart tissue from SAV infected, PRV infected and PRV-SAV co-infected fish were stained using polyclonal rabbit antiserum targeting PRV σ1 (left panel) and monoclonal murine anti-SAV E2 (right panel). The fish were sampled at the same time point, 7 WPC-PRV which corresponds to 3WPC-SAV in the early co-infection. The small internal panels are enlargements to show specificity of the staining.

neighboring cells in heart tissue by immunohistochemistry along with co-detection by RTqPCR. However, since the protective effect was observed in pancreas which is not a target organ for PRV, this indicates that the PRV mediated protection is due to systemic responses and not to interaction through co-infection in the same tissue. PRV utilizes erythrocytes for replication and dissemination in the fish [34], and the salmon erythrocytes can mount innate antiviral responses after PRV infection [12, 43].

The PRV control group had a significantly higher k-factor 10 WPC-PRV compared to the SAV control groups at the same time point. The k-factors in the SAV2 and SAV3 controls were lower compared to the corresponding double infected groups (PRV-SAV2-early and PRV-SAV3-early), although only significant for the PRV-SAV3-early group. The higher k-factor supports the observed less severe histopathological changes in the co-infected groups, confirming the reduced impact of SAV after a preceding PRV infection.

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Table 2 Fold expression levels relative to naïve fish at day 0 of selected genes from microarray analysis at 4 or 6 WPC-SAV in PRV-SAV3-late and SAV3 control groups

Gene	PRV-SAV3-late	SAV3 control	Difference	*p* value
4 WPC-SAV				
Serum amyloid A-5 protein	2.79	4.15	−1.36	0.212
Interleukin 1-receptor accessory protein-like 2	1.29	4.26	−2.98	0.040
Matrix metalloproteinase 13	0.97	5.67	−4.69	0.015
Calsequestrin	0.62	−3.02	3.63	0.003
Neuropeptide Y-1	0.33	2.85	−2.53	0.002
Arginase-1	0.03	−1.95	1.98	0.001
Arginase-2, mitochondrial	0.80	2.60	−1.79	0.065
6 WPC-SAV				
Serum amyloid A-5 protein	1.67	4.98	−3.31	0.001
Interleukin 1-receptor accessory protein-like 2	0.36	3.89	−3.53	0.001
Matrix metalloproteinase 13	0.18	5.43	−5.25	0.001
Calsequestrin	0.29	−1.68	1.97	0.001
Neuropeptide Y-1	0.08	2.99	−2.91	0.001
Arginase-1	0.01	−1.51	1.52	0.001
Arginase-2, mitochondrial	0.11	3.17	−3.06	0.001

Our results are in accordance with previous co-infections in fish where reduced mortality was observed. Pre-exposure of rainbow trout with either the non-virulent cutthroat trout virus (CTV) (*Hepeviridae*), chum salmon reovirus (CSV), or IPNV, gave a four week protection to a subsequent IHNV infection [26–30, 44, 45]. A similar effect against ISAV, lasting eight weeks, was observed for IPNV infected fish. However in that study the ISAV challenge was given intraperitoneally [46]. This indicates that long-lasting cross-protection between non-related viruses in fish is a general phenomenon, although the duration of protection may vary. Our study suggest an inhibitory effect of PRV on a secondary SAV infection which may last for at least 10 weeks post PRV challenge, and thus a longer duration of protection compared to other interfering virus infections reported in salmonids [26, 46].

Activation of the antiviral innate immune response, where the type 1 interferon (IFN) system is central [47, 48], is a possible explanation for protection against unrelated viruses after a primary virus infection. Studies on SAV infection in cell cultures and in vivo have shown upregulation of IFNα and a number of interferon induced genes [49, 50]. However, these studies revealed a complex antiviral innate response after SAV infection leading to reduced SAV propagation. PRV infection of Atlantic salmon RBC also cause strong up-regulation of antiviral genes of the innate immune response [43]. Two recent studies show that Mx expression is upregulated in heart tissue for 11 weeks [41], and in erythrocytes for at least eight weeks after PRV infection [43]. Interferon type I production was induced at the transcriptional level in erythrocytes for up to 7 weeks [43]. This suggests that circulating PRV infected erythrocytes could play an important role in the observed suppressive effect on SAV propagation and PD development by inducing interferon-regulated antiviral responses in most organs prior to SAV infection [51].

Therefore, at the time of early SAV shedder introduction in our trial, i.e. at 4 WPC-PRV, an upregulation of innate antiviral genes is expected. A possible long lasting innate immune response may contribute to the protection seen during the late co-infection. SAV RNA levels in heart were significantly lower 6 WPC-SAV in the early co-infected groups compared to the SAV controls. This difference was not present at the same time point in the late co-infected hearts, which could indicate a decreased protection caused by a reduction of innate immunity. However, since fish IgM have lower specificity and antigen affinity compared to mammalian serum antibodies, at least up to 15 weeks post infection, a mechanism of low affinity polyreactive natural antibodies cannot be ruled out [52, 53].

A possible difference was observed between the two SAV subtypes in the ability to handle the consequences of the preceding PRV infection. The SAV2 and SAV3 RNA levels differed in the co-infected groups. SAV2 replicated more efficiently than SAV3 during the early co-infection, whereas the peak phase of both viruses was lost in the late co-infection with PRV, either by a delay (SAV2) or by a reduction (SAV3). There were large individual variations in levels of SAV RNA and in prevalence of SAV
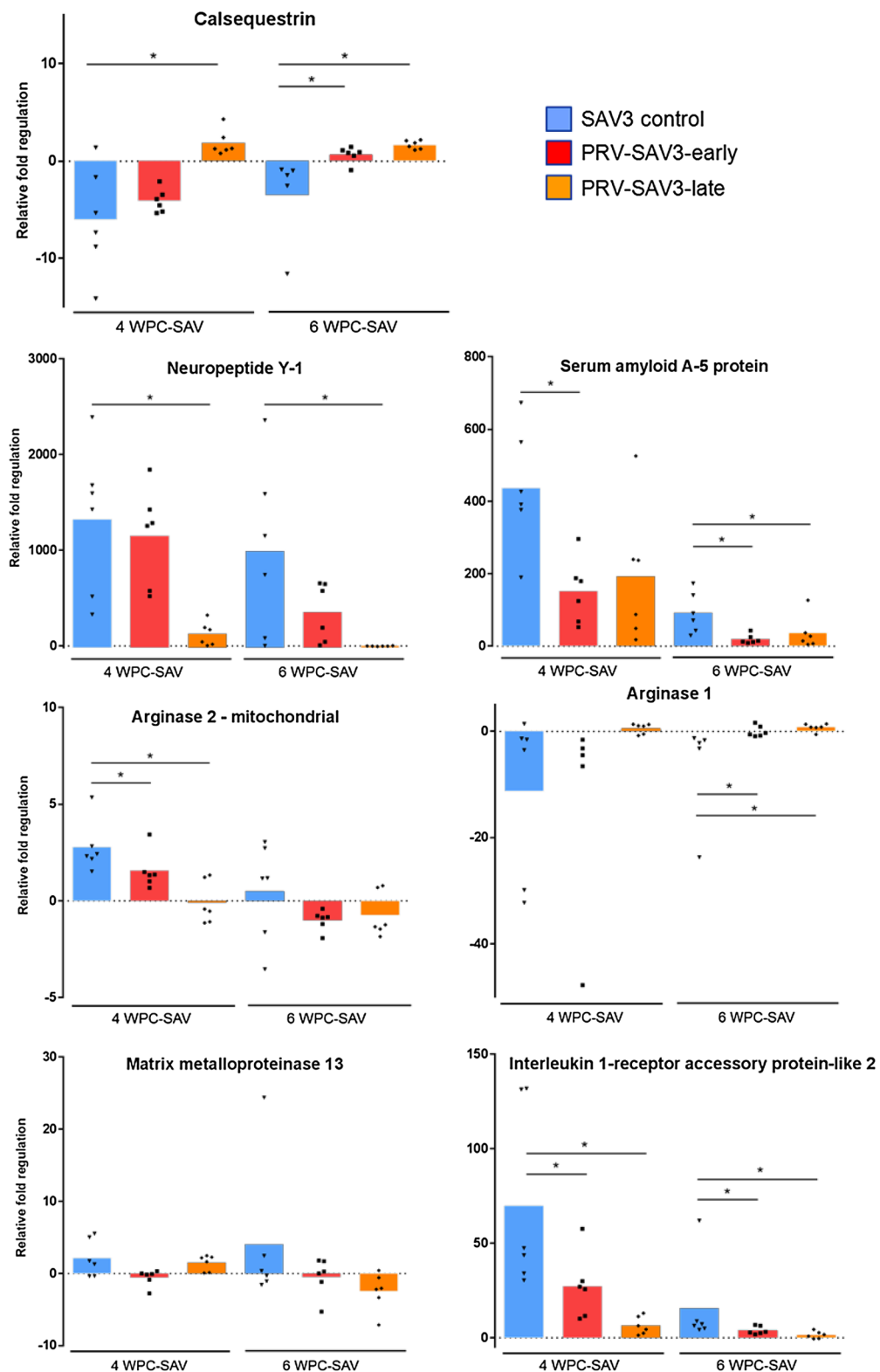
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Figure 9 Expression of potential pancreas disease marker genes in heart. Fold induction or repression of genes identified as potential PD associated genes assessed in SAV3 infected (blue bars) and co-infected (red bars: PRV-SAV3-early, orange bars: PRV-SAV3-late) 4 or 6 weeks after SAV3 shedder introduction. Significant differences between SAV3 controls and co-infected groups are indicated with *$p < 0.05$. Boxes indicate mean fold change relative to mean levels at 4 WPC-PRV.

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Table 3 Spearman's rank correlation between gene expression (RTqPCR) and histopathology score of inflammation in myocardium and acute necrosis in myocardium

Spearman's rank correlation

	Myocardial degeneration and inflammation		Acute myocardial necrosis	
	r_s	*p* value	r_s	*p* value
Matrix metalloproteinase 13	0.0301	0.8427	0.3573	0.0148
Calsequestrin	−0.5284	0.0002	−0.4372	0.0024
Interleukin 1-receptor accessory protein-like 2	0.527	0.0002	0.7391	<0.0001
Neuropeptide Y-1	0.7049	<0.0001	0.6288	<0.0001
Serum amyloid A-5 protein	0.341	0.0204	0.7386	<0.0001
Arginase-2, mitochondrial	0.3798	0.0092	0.7135	<0.0001
Arginase-1	−0.6536	<0.0001	−0.4278	0.0030

positive fish in the co-infected groups, which makes it difficult to conclude if the apparent differences in SAV2 and SAV3 kinetics show true different properties of the virus subtypes.

SAV RNA kinetics in the heart tissue of both SAV2 and SAV3 controls were similar when assessed by RTqPCR, which is in accordance with a previous SAV challenge trial where several isolates were tested [21]. An interesting finding, that should be addressed in a suitable study, was the strong correlation between acute myocardial necrosis and SAV3 RNA level ($r_s = 0.81$) compared to SAV2 ($r_s = 0.59$) in the individual infected fish. If SAV3 is a stronger inducer of acute myocardial necrosis, this may partly explain the observed higher mortality associated with SAV3 compared to SAV2 [21, 22, 54].

A previous study using salmon microarray and RTqPCR analysis have reported that changes in the expression levels of certain genes are specifically associated with SAV mediated pathological changes in heart [41]. We found a similar regulation of these genes in the SAV controls in our study and that the expression levels of these genes were less affected in the co-challenge groups. This is in tune with the protective effects of a primary PRV infection, supporting histopathological observations and virus kinetics. RTqPCR run on a selected number of genes confirmed the microarray results. In general, the gene expression pattern was more affected in the SAV controls compared to the co-infected group. A previous study indicated a difference in gene expression patterns between the two diseases [41]. Our study found that the expression differences between the

co-infected and SAV3 control groups changed in line with score of histopathological lesions, with a strong correlation of neuropeptide Y-1 and arginase-1 expression to the score of myocardial degeneration and inflammation. In mammals, neuropeptide Y has been shown to have several effects on inflammatory responses and cardiomyopathy [55, 56]. Furthermore, expression of IL1R-2, SAA5 and mitochondrial arginase-2 show a strong and significant correlation towards acute myocardial necrosis. Thus, genetic analysis may prove to be an additional tool for evaluation of the severity of salmon heart disease and tissue damage.

A peculiar finding was the secondary PRV infection detected in the SAV shedders who resided with the PRV cohabitants. This was observed in both the early and late co-infection. During the early co-infection, SAV2 shedders had a higher PRV RNA level, although not significant, in heart when compared to SAV3 shedders six weeks after introduction to PRV cohabitants. This may suggest that SAV3 yields a stronger protection against PRV than SAV2. A possible explanation is that SAV3, reported by others to be more virulent [21, 22, 54], yield a stronger immune response than SAV2. However, the lack of parallel tanks and number of fish per group ($N = 6$) must be accounted for when interpreting these results. The strong correlation between SAV RNA levels and myocardial necrosis may be a novel step towards understanding the observed virulence differences between the subtypes. An interesting observation in this perspective is the higher prevalence of HSMI in the geographically separated endemic areas of SAV2 in mid-Norway compared to those of SAV3 further south [4, 7]. These field observations could potentially be linked to a higher possibility of cross-infection between SAV2 and PRV and more prevalent development of both diseases in PRV-SAV2 dual infected fish.

Interactions between viral diseases may be part of the explanation for the large variation in severity described for SAV infections in the field [24]. PRV is found to infect fish in fresh water facilities and is ubiquitous in sea farms [6]. The protective effect in this study could affect the outcome of a PRV-SAV co-infection after sea transfer. In this study, the experimental fish had high levels of PRV RNA and developed HSMI. A more subtle PRV infection, where there is no HSMI development may cause a difference in strength and duration of the protection. Further research should address various field conditions when assessing the implications of PRV-SAV co-infection.

In conclusion, we found that a primary PRV infection partially protects against the outcomes of SAV infection and PD pathological lesions.

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Additional files

Additional file 1. Scoring criteria for histopathological changes.
The table explains the categories of inflammatory changes that was used in the histopathological scoring.

Additional file 2. Mean weight and k-factor is presented with range in brackets. Accumulated mortality is presented in N with% in brackets. Number indicates weeks post PRV challenge. PRV-SAV2/3e and PRV-SAV2/3l indicates early and late co-infection, respectively.

Additional file 3. Histopathology evaluation of epicarditis, pancreas pathology, acute necrosis in myocardium, inflammation in myocardium, inflammation in white and red skeletal muscle in PRV cohabitants. Weeks after PRV challenge is shown. Week 0 represents uninfected control fish.

Additional file 4. Immunohistochemistry of co-infected heart tissue stained using polyclonal rabbit antiserum targeting PRV σ1 (right panel) and monoclonal murine anti-SAV E2 (left panel). The heart tissue had Ct values of 14.2 and 15.9 for SAV and PRV, respectively.

Additional file 5. Immunohistochemistry of SAV-infected heart tissue stained using polyclonal rabbit antiserum targeting PRV σ1 (right panel) and monoclonal murine anti-SAV E2 (left panel). The heart tissue had a SAV Ct-value of 17.1.

Additional file 6. Immunohistochemistry of PRV infected heart tissue stained using polyclonal rabbit antiserum targeting PRV σ1 (right panel) and monoclonal murine anti-SAV E2 (left panel). The heart tissue had a PRV Ct- value of 17.7.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MKD, VA and ER launched the project idea. MKD and ER participated in the overall design and coordination of the study, interpretation of data and drafting the manuscript. AK and GT performed and interpreted the data from the micro array and revised the manuscript. IBN performed gene expression analysis and revised the manuscript. VA participated in the coordination of the study and revised the manuscript. ML coordinated the challenge design and study, all practicalities regarding sample logistics, gathering and interpretation of data and drafted the manuscript. MVR coordinated the challenge design and study, carried out the histopathological scoring, performed immunohistochemical staining, interpretation of data and drafted the manuscript. All authors read and approved the final manuscript.

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Paper III

Immunological interactions between *Piscine orthoreovirus* and *Salmonid alphavirus* infections in Atlantic salmon

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Abstract

Heart and skeletal muscle inflammation (HSMI) and pancreas disease (PD) cause substantial losses in Atlantic salmon (*Salmo salar*) aquaculture. The respective causative agents, *Piscine orthoreovirus* (PRV) and *Salmonid alphavirus* (SAV), are widespread and often concurrently present in farmed salmon. An experimental infection in Atlantic salmon was conducted to study the interaction between the two viruses, including the immunological mechanisms involved. The co-infected fish were infected with PRV four or ten weeks before they were infected with SAV. The SAV RNA level and the PD specific lesions were significantly lower in co-infected groups compared to the group infected by only SAV. The expression profiles of a panel of innate antiviral response genes and the plasma SAV neutralization titers were examined. The innate antiviral response genes were in general upregulated for at least ten weeks after the primary PRV infection. Plasma from co-infected fish had lower SAV neutralizing titers compared to the controls infected with only SAV. Plasma from some individuals infected with only PRV neutralized SAV, but heat treatment removed this effect. Field studies of co-infected fish populations indicated a negative correlation between the two viruses in randomly sampled apparently healthy fish, in line with the experimental findings, but a positive correlation in moribund or dead fish.

The results indicate that the innate antiviral response induced by PRV may temporary protect against a secondary SAV infection.

Introduction

Piscine orthoreovirus (PRV) was first described in association with heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (*Salmo salar*) in 2010 [1]. PRV clusters within the genus *Orthoreovirus* in the family *Reoviridae,* which are non-enveloped viruses with a genome consisting of ten segments of double-stranded RNA [1–3]. Erythrocytes are important target cells for PRV, but the virus also infects cardiomyocytes and red skeletal muscle cells [4,5], and the inflammation in heart and red skeletal muscle named the disease [6]. Although PRV is detected in both clinically healthy and HSMI diseased farmed Atlantic salmon, there is a correlation between HSMI and high load of viral RNA in hearts [7,8]. The prevalence of PRV positive salmon during the production cycle varies, but the vast majority of sea reared farmed Atlantic salmon becomes infected during the production [7].

The high prevalence of PRV infection in farmed Atlantic salmon in seawater makes the likelihood for co-infections with other viruses plausible. Co-infections between PRV and *Salmon pancreas disease virus* (SPDV) and between PRV and *Piscine myocarditis virus* (PMCV) have been described [8–10]. SPDV, more commonly known as salmonid alphavirus (SAV), is the etiological agent of pancreas disease (PD) in Atlantic salmon, and it causes significant economic losses [11–14]. In this paper, we further use SAV. Six subtypes of SAV have been described [15], of which subtypes 2 and 3 are endemic in Norway [16,17].

A primary PRV infection reduced the effect of a subsequent SAV infection in an experimental challenge by reducing the prevalence and severity of the SAV infection at four and ten weeks after the initial challenges [18]. This is in contrast with a recent finding where primary PRV infection had no effect on a secondary infection with infectious hematopoietic necrosis virus (IHNV), conducted two weeks after the PRV infection in Sockeye salmon (*Oncorhyncus nerka*) [19]. Various cross protection between viral infections in fish has been demonstrated, although with differences in challenges settings and in duration of the protection [20–29].

PRV infection in Atlantic salmon upregulates genes of the innate antiviral immune response, including the IFN cascade, and down-regulates a large part of non-immune related genes [30]. A study on Atlantic salmon post-smolts demonstrated a coinciding peak expression of Mx and PRV RNA in hearts [31] (PRV was called Atlantic salmon reovirus in that study), which has also been supported by other findings [32,33]. Together, these studies indicate a strong innate immune response induction by PRV infection in Atlantic salmon.

In this study, using the subtype SAV2, the mechanisms of the PRV-SAV cross-protection were targeted. We investigated the expression of innate antiviral immune genes and the SAV neutralizing titer of plasma in experimentally PRV-infected and PRV-SAV co-infected fish. To investigate if the experimental cross protection could be detected under farming conditions two different datasets with correlation analysis of the two viruses was included.

Materials and methods

Challenge trial

The samples were collected in a challenge trial described in detail earlier [18]. Briefly, seawater adapted Atlantic salmon post-smolts were challenged with PRV by cohabitation in a 1:1, shedder:cohabitant, ratio (Figure 1). The PRV shedders were removed after four weeks and the cohabitants were distributed in two tanks. PRV cohabitants in one tank were exposed to SAV shedders at 4 weeks after PRV challenge (SAV-early), while the fish in the remaining tank were exposed to SAV shedders 10 weeks after PRV challenge (SAV-late). The PRV-SAV co-infections lasted for 6 weeks and sampling was performed regularly. In the original challenge trial both SAV subtypes 2 and 3 were studied in parallel. For clarity and relevance to field samples, only the SAV2 groups are included in the present work, and hence SAV addresses only SAV2. The challenge trial was approved by the Norwegian Animal Research Authority, and performed in accordance with the recommendations of the current animal welfare regulations: FOR-1996-01-15-23 (Norway).
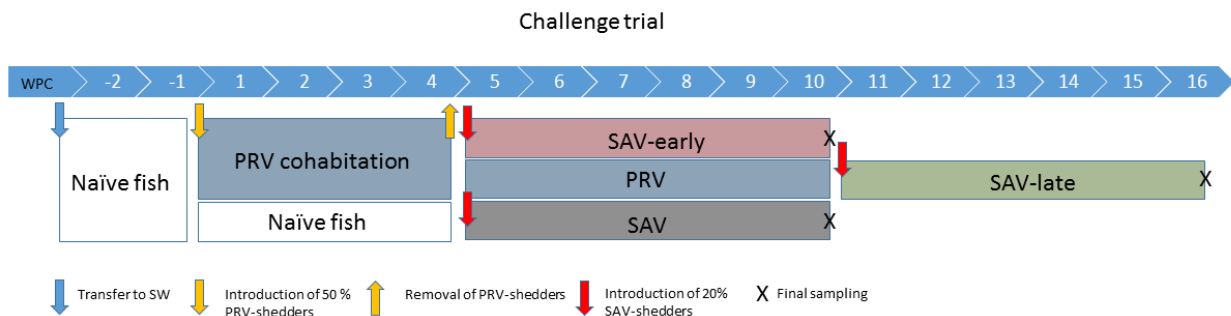

Figure 1. *The challenge trial. Timeline indicates weeks post introduction of PRV shedders (WPC-PRV). Naïve fish were transferred to seawater and acclimatized for two weeks before 50% were injected to become PRV-shedders. The PRV shedders were removed after four weeks and 20% SAV shedders were added to one tank (i.e. the SAV-early group). To the remaining PRV cohabitant group, 20 % SAV-shedders were added at 10 WPC-PRV (i.e. the SAV-late group). In addition, one group was infected with only SAV, this group was called the SAV group.*

Sampling

Samples from heart and spleen were collected in 0.6 ml RNA*later*™ (Ambion Inc., USA) in prefilled 1.0 ml tubes (FluidX® Ltd, UK). Blood was collected from the caudal vein on

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heparinized vacutainer tubes (Fisher Scientific) and centrifuged (850 x g, 10 min, 4°C). Plasma was separated from the blood cell pellet and both were stored at -80°C.

Gene expression by real time qPCR

RNA extraction from heart, spleen and blood cell pellet (N=8 per time point) and subsequent RTqPCR were performed for PRV, SAV and elongation factor 1α (EF1α), using assays described earlier [34–36]. RNA quantity was determined by using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNase Out (Life technologies) was added to a final concentration of 0.4 U/µl and the RNA was stored at -80°C awaiting gene expression analysis. cDNA was synthetized from 600 ng total RNA using QuantiTect Reverse Transcription Kit (Qiagen) containing gDNA wipeout buffer according to the manufacturer's instructions. A representative sample mix was prepared for cDNA synthesis with and without addition of RT-enzyme, and used for assessing efficiency and control background contamination of genomic DNA for all assays prior to the analysis of individual samples. Quantitative PCR was performed using 15 ng (5 µl of 3 ng/µl) cDNA input per reaction. The genes targeted for expression analysis, primer and probe sequences and their origins, are shown in Table 1. For new primers targeting genes containing introns, one of the primers was designed to cross exon-exon junctions. Both Maxima SYBR Green (Thermo Scientific) with 10 µM of both primers and QuantiFast Probe (10 µM) PCR + ROX Vial kit (Qiagen) were used. The cyclic conditions were 95°C for 10 min, then 40 cycles of 95°C/15 sec, 60°C/30 sec and 72°C/30 sec in a Mx3005P (Stratagene, La Jolla, CA, USA) for all analyses. Melting curve analyses were performed for each SYBRGreen assay. All samples were run in duplicates on the same plate for each qPCR assay. A seven-point concentration grade standard curve (40-0.675 ng) was run for validation of the primer pairs.

Table 1. *Primers, probes, amplicon lengths, references and GenBank accession numbers for genes targeted.*

Gene	Nucleotide sequences	Amplicon length	Reference	GenBank no.
Elongation factor 1αB **EF1α**	Fwr: 5'TGCCCCTCCAGGATGTCTAC'3 Rev: 5'CACGGCCCACAGGTACTG'3	57	[36]	XM_014141923
β-actin	Fwr: 5'CCAAAGCCAACAGGGAGAAG'3 Rev: 5'AGGGACAACACTGCCTGGAT'3 Probe: 6FAM-TGACCCAGATCATGTTT-MGBNFQ	91	[36]	BG933897
Interferon a **IFN a**	Fwr: 5'ACTGAAACGCTACTTCAAGAAGTTGA'3 Rev: 5'GCAGATGACGTTTTGTCTCTTTCCT3'	104	[37]	AY216595 AY216594

Hepcidin	Fwr: 5'ATGAAGGCCTTYAGTGTTGCAGT'3	99	New	XM_014170058
	Rev: 5'TCCGTTCGCACCTCGGAGAA'3			
β-defensin	Fwr:5'GAGGCTGCATCATTTCCCTTCTCTT'3	123	New	FN545575
	Rev:5'ATGAGAAACACAGCACAAGAATCCCTTT'3			
Cholesterol 25-hydroxylase CH25H	Fwr: 5'GCTGGCCTGGATCAGGACCTTT'3	127	New	XM_014178944
	Rev: 5'GTGCAGCACAGTGAGAGGGAAGA'3			
Ubiquitin specific peptidase 18 USP18	Fwr: 5'GCTACTAGCCATGCAGAGTGACCAA'3	176	New	XM_014153344
	Rev: 5'GAATCTCCTGAGCCAAGGCCTTGT'3			
Retinoic acid-inducible gene I RIG-1	Fwr: 5'GCGACCGTCTTACGTCAAAG'3	112	[38]	FN178459
	Rev: 5'TAGAAACACCTGGGCTGCTG'3			
Myxovirus resistance gene Mx	Fwr: 5'GGTGATAGGGGACCAGAGT'3	173	New	BT043721.1
	Rev: 5'CTCCTCACGGTCTTGGTAGC'3			
Protein kinase R PKR	Fwr: 5'CAGGATGCAACACCATCATC'3	162	[38]	EF523422.1
	Rev: 5'GGTCTTGACCGGTGACATCT'3			
Interferon-stimulated gene 15 ISG15	Fwr:5'ATATCTACTGAACATATATCTATCATGGAACTC'3	150	New	AY795563
	Rev: 5'CCTCTGCTTTGTTGTGGCCACTT'3			
Viperin	Fwr:5'AGCAATGGCAGCATGATCAG'3	101	[39]	NM_001124253
	Rev: 5'TGGTTGGTGTCCTCGTCAAAG'3			

SAV neutralizing assay

A SAV neutralization assay was performed for plasma samples. After thawing, the plasma samples were split into two equally large aliquots. Complement was inactivated in one aliquot by heating (48°C for 20 minutes) as described by Lamas *et al* [40]. The assay was performed with chinook salmon embryo (CHSE-214) cells as earlier described [41]; the method was modified to detect SAV antigens with immunofluorescence after acetone fixation. When a neutralizing effect was observed at a dilution of 1:20, endpoint titration was performed.

Field study

The first field sample series were heart samples on RNA*later*™ from dead and moribund fish (N = 307), collected from ten different Atlantic salmon sea farm sites in the SAV2 endemic region of Norway. The samples were collected during the years 2011– 2014 at 16 time points as part of the company's surveillance program and health inspections. Only sampling points with detection of both SAV and PRV were included. This collection of samples and the respective data are referred to as "Screening data".

The second sample series originated from a longitudinal observational study performed at one sea site that received smolts in June 2014. Screening for SAV was performed by RTqPCR monthly (N=20). HSMI was diagnosed in the population December 2014. The first SAV detection at the sea site was late April 2015. After this time-point, randomly selected fish from two study pens were sampled more frequently. Four sampling points were included;

Sampling 1 (N=40) at the time of first detection of SAV in the study pens (June 2015); Sampling 2 (N=39) three weeks after Sampling 1, at the assumed peak level of SAV infection; Samplings 3 (N=40) and 4 (N=40) at 7 and 11 weeks after Sampling 1, respectively. Before sampling, the fish were euthanized by an overdose of sedative (Benzoak vet., ACD Pharmaceuticals AS, Norway). Tissue samples of heart (N=159) were collected on RNA*later™*. The data generated from this sample series are addressed as "Longitudinal study".

PatoGen AS (Ålesund, Norway) performed the RT-qPCR for PRV and SAV in all field samples. The threshold for assigning a sample negative for PRV or SAV was Ct value $\geq$ 37.0. RT-qPCR for EF1α was performed on all samples and samples having an EF1α Ct > 27.0 were excluded due to assumed RNA degradation.

Data analysis and statistics
The RTqPCR data for gene expression in heart tissue are presented as $2^{-\Delta\Delta Ct}$ with EF1α as the reference gene and mean expression at Day 0 (uninfected) as the reference time-point. Viral RNA and gene expression transcripts in blood and spleen are presented as Ct values due to significant regulation of the reference gene.

A nonparametric approach was used for statistical analyses of differences from Day 0 in the challenge trial. The Wilcoxon rank-sum were used to test for time-point differences in gene transcription levels between groups. Spearman's correlation assessed the relationship between viral transcripts of PRV and the genes of interest using Ct values for all three organs.

The results from the Screening data and the Longitudinal study was handled separately. A Spearman's correlation coefficient (r) assessed the relation between PRV and SAV RNA levels.

Results

Challenge trial
The challenge trial was a cohabitation model where a primary PRV infection lasted four or ten weeks (WPC-PRV) before SAV-shedders were introduced. The levels of PRV RNA in spleen after the primary PRV infection are displayed in Figure 2A. The large variation in PRV RNA levels at 4 WPC-PRV probably reflects different stages in the individual fish in this phase of the infection. PRV RNA levels in spleen peaked at 7 WPC-PRV and displayed a weak decline 10 WPC-PRV. The group co-infected with SAV at 4 WPC-PRV (SAV-early) had significantly lower levels of SAV RNA compared to the SAV control groups at 3, 4 and 6

weeks post SAV infection (Figure 2B). The fish co-infected at 10 WPC-PRV (SAV-late) also had lower SAV RNA levels compared to the controls at week 2, 3 and 6 after SAV infection. However, the SAV-late group had higher levels of SAV RNA 4 weeks post SAV infection (Figure 2B). There is a time difference between SAV- control and SAV-late of six weeks that should be taken into account when interpreting SAV- late group The results indicated lower prevalence and levels of SAV RNA in spleen of the co-infected fish compared to those infected with SAV only.
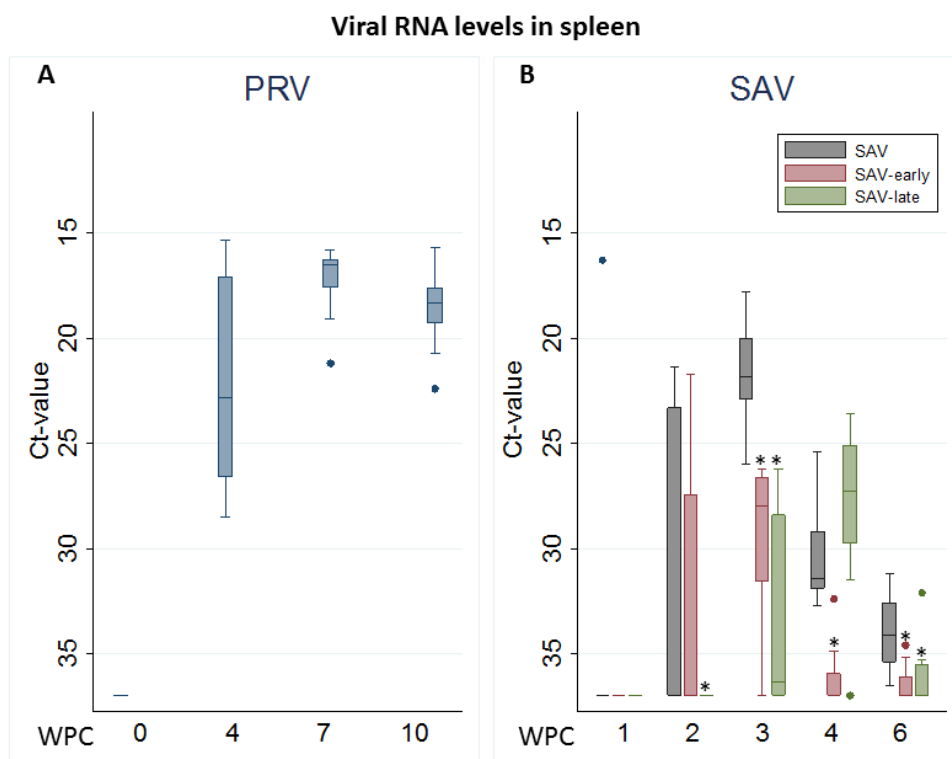
Figure 2. *Viral RNA levels in spleen. **A**: PRV RNA levels in PRV infected fish 0, 4, 7 and 10 WPC-PRV. **B**: SAV RNA levels in SAV control and in PRV-SAV co-infected groups, SAV-early (4 WPC-PRV) and SAV-late (10 WPC-PRV) at 1, 2, 3, 4 and 6 weeks post SAV infection. Results are presented as box plot with a horizontal line within each box indicating the median. The box regions are limited by 25th percentile and 75th percentile, and whiskers mark the upper and lower adjacent value (1.5*inter-quartile range). Outliers are marked with dots. * indicates significant difference from SAV control ($p < 0.05$).*

Normalization of gene expression analysis

The commonly used reference gene EF1α was not stably expressed in all organs during the experiment (Figure 3). EF1α expression levels were stable in heart tissue, but displayed significant variation in blood and spleen at 4 and 10 WPC-PRV compared to Day 0 ($p < 0.05$) (Figure 3A). Similarly, expression levels of the β-actin reference gene in heart (n=4 at 0 WPC) and blood cells also differed significantly between Day 0 and 10 WPC-PRV, and in blood cells also at 4 WPC-PRV (Figure 3B). There was a positive correlation between Ct values of EF1α and PRV in blood cells and spleen, but not in heart samples (Table 2). A positive correlation was also found between β-actin Ct values and PRV Ct values in blood cells. Based on the significant regulation of EF1α and β-actin together with the positive correlation with PRV. Gene expression transcript from blood and spleen is presented as Ct values.
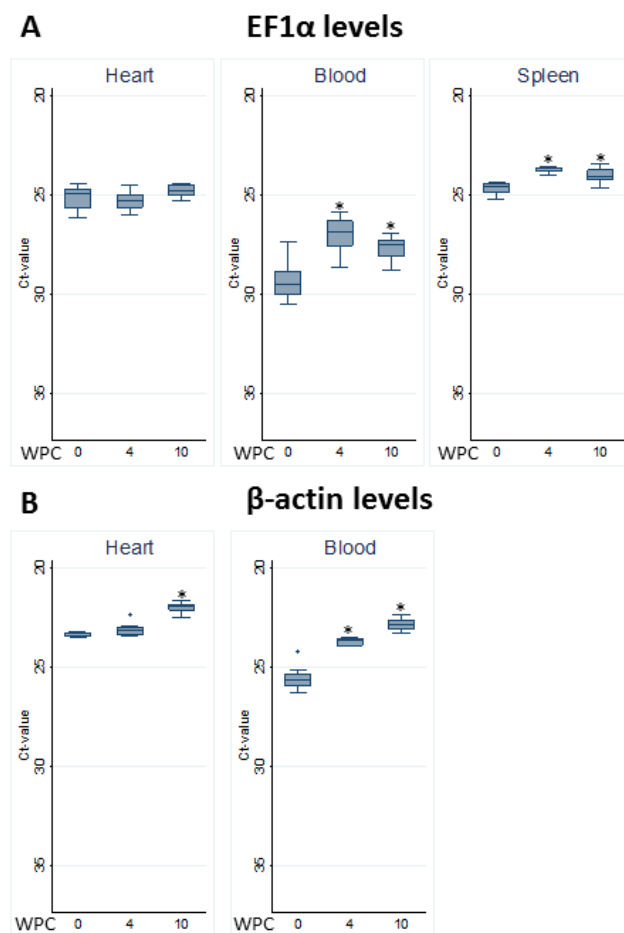
Figure 3. *Expression of EF1α and β-actin in investigated tissue at 0, 4 and 10 WPC-PRV. n= 8 at each time point, except for 0 WPC heart for β-actin n=4. Results are presented as box plot a horizontal line within each box indicating the median. The box region are limited by*

25th percentile and 75th percentile, and whiskers mark the upper and lower adjacent value (1.5*inter-quartile range). Outliers are marked with dots. * indicates significant difference from 0 WPC-PRV (p<0.05).

Table 2. *Spearman's rank correlation between Ct-values of gene transcripts and PRV RNA in blood, spleen and heart, over all sampling points (0, 4 and 10 WPC-PRV). * indicates significant correlation (p < 0.05). - = Not performed.*

Spearman's correlation			
	Blood	Spleen	Heart
Ef1α	0.58*	0.62*	-0.20
B-actin	0.58*	-	0.15
CH25H	-0.36	0.46*	0.00
IFN	0.55*	0.84*	0.90*
β-defensin	0.34	-0.33	-0.52*
Hepcidin	0.10	0.27	-0.40
ISG15	0.79*	0.85*	0.79*
Mx	0.59*	0.87*	0.77*
PKR	0.78*	0.87*	0.90*
RIG	0.75*	0.87*	0.88*
Viperin	0.80*	0.92*	0.87*
USP18	0.71*	0.86*	0.74*

Expression of innate immune genes

The expression patterns of innate antiviral immune genes were examined in PRV-infected samples at the time when SAV shedders were introduced, i.e. at 4 WPC-PRV (SAV-early) and 10 WPC-PRV (SAV-late), respectively. PRV infection induced increased expression of the innate antiviral immune genes IFN-I, Mx, Viperin, ISG15, PKR and RIG in blood cells, spleen and heart (Figure 4). At 4 WPC-PRV, large individual variations in the PRV RNA level and the innate antiviral immune gene expression were observed, in contrast to 10 WPC-PRV when this variation was smaller (Figures 2 and 4).

In the heart, CH25H and UPS18 were not significantly upregulated at 4 WPC-PRV, while at 10 WPC-PRV all genes investigated were upregulated in heart (Figure 4).
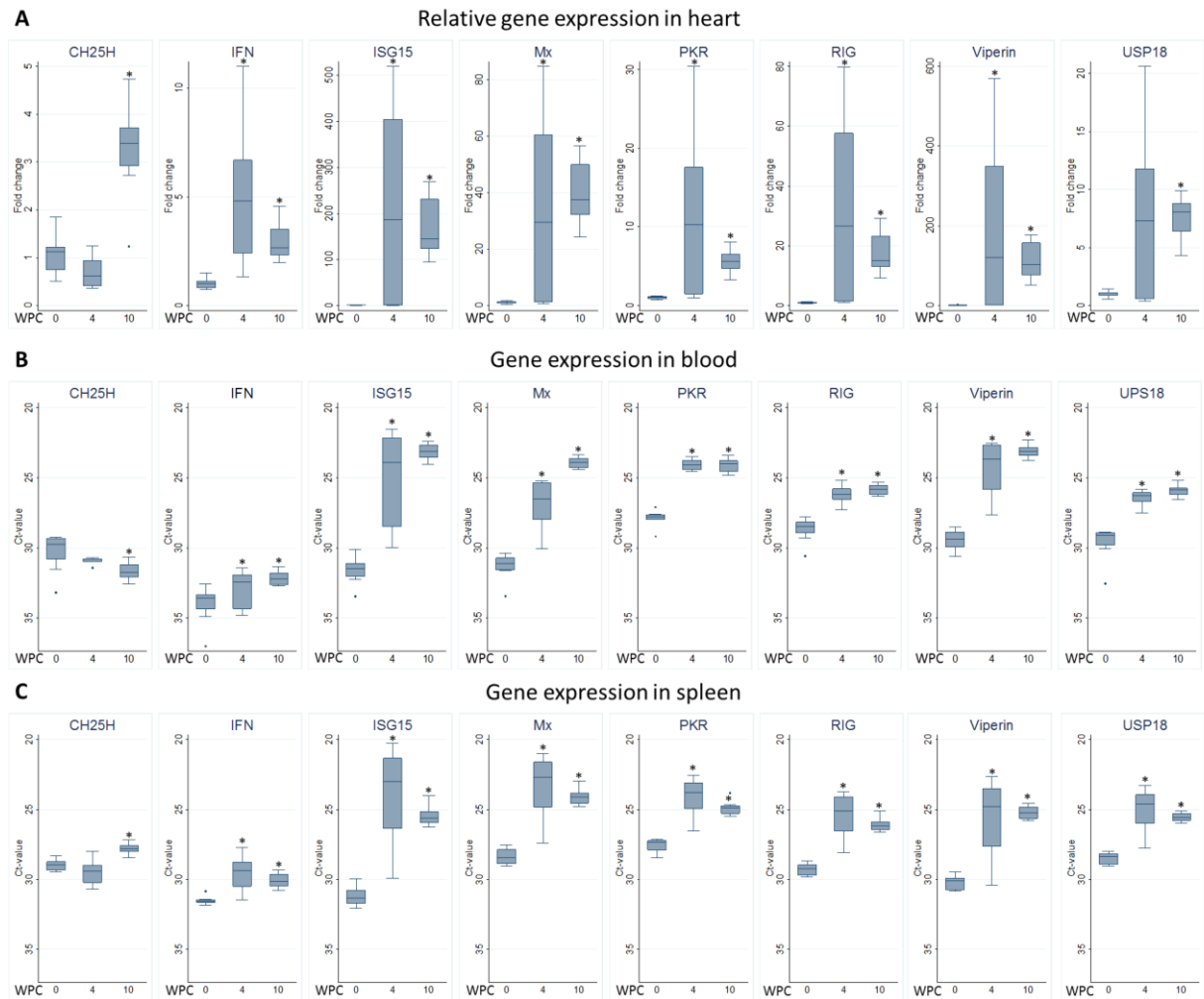
Figure 4. *Expression of antiviral innate immune genes in heart, blood and spleen tissue of PRV cohabitant group. Samples from heart (A) is presented as fold expression using EF1α as reference gene with mean expression at 0 WPC as a baseline. Samples from blood (B) and spleen (C) is presented as Ct-values, due to significant regulation of EF1α. The middle bars (lines within each box) represent the median, box region are limited by 25th percentile and 75th percentile, and whiskers marks the upper and lower adjacent value (1.5*inter-quartile range). Outliers are marked with dots. * indicates significant difference from 0 WPC (p<0.05).*

The same pattern was present in blood and spleen, with the exception of CH25H (Figure 4). This gene showed reduced transcription in blood cells at both 4 and 10 WPC-PRV, being significant at 10 WPC-PRV ($p < 0.05$). In spleen and heart however, there was a significant upregulation at 10 WPC-PRV ($p < 0.05$). The correlations between levels of viral RNA and

innate immune gene expression are displayed in Table 2, showing that activation of innate antiviral immune response cascades correspond well with PRV RNA levels.

Antimicrobial peptides

The transcription of β-defensin and hepcidin genes in heart, spleen and blood are displayed in Figure 5. There was a significant upregulation of both β-defensin and hepcidin genes in blood cells at 4 WPC-PRV compared to Day 0 ($p < 0.05$), however the contrary was detected at 10 WPC-PRV. In spleen, there was a significant increase in hepcidin gene expression at 4 WPC-PRV ($p < 0.05$) and no difference at 10 WPC-PRV. Expression of β-defensin in spleen was low at all time-points investigated. In heart tissue, the level of β-defensin gene expression was significantly down-regulated at both time points ($p < 0.05$), however no differences in hepcidin levels were detected. It should be noted that β-defensin expression was negatively correlated to PRV RNA levels in heart tissue (Table 2).
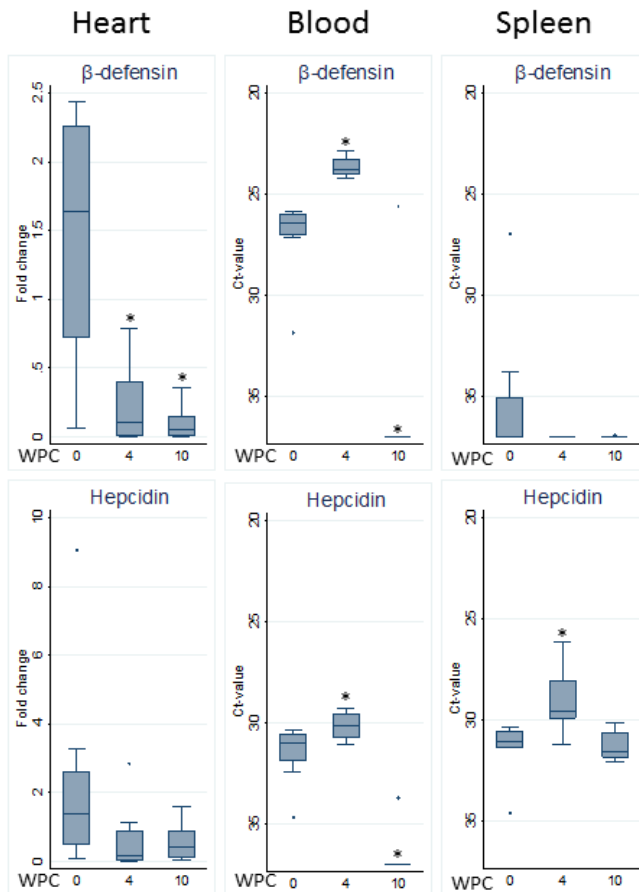
Figure 5. *β-defensin and hepcidin RNA levels in heart, blood and spleen. EF1α was used as reference gene for heart samples using mean expression at Day 0 as baseline. Results from blood and spleen are displayed as Ct-values. The middle bar represent the median, box region are limited by 25th percentile and 75th percentile, and whiskers marks the upper and lower adjacent value (1.5*inter-quartile range). Outliers are marked with dots. * indicates significant difference from 0 WPC (p < 0.05)*

Serology

The SAV neutralizing activity increased with time in the plasma of the SAV infected control group. After 6 weeks of SAV infection, plasma from all SAV controls exhibited neutralizing activity, regardless of heat inactivation of complement (Figure 6A). In the PRV infected control group, plasma from four individuals neutralized SAV at 4 WPC-PRV before heat treatment (N = 16) (Figure 6B). The neutralization was strong (1:160) in one sample and weak (1:20) in three samples. After heat treatment, the plasma of the individual showing strongest neutralization still had a neutralizing effect (1:80) (Figure 6B). No neutralization of plasma from the PRV group was observed at 7 and 10 WPC-PRV (Figure 6B). In the co-infected fish, neutralizing activity was first detected two weeks after SAV infection.
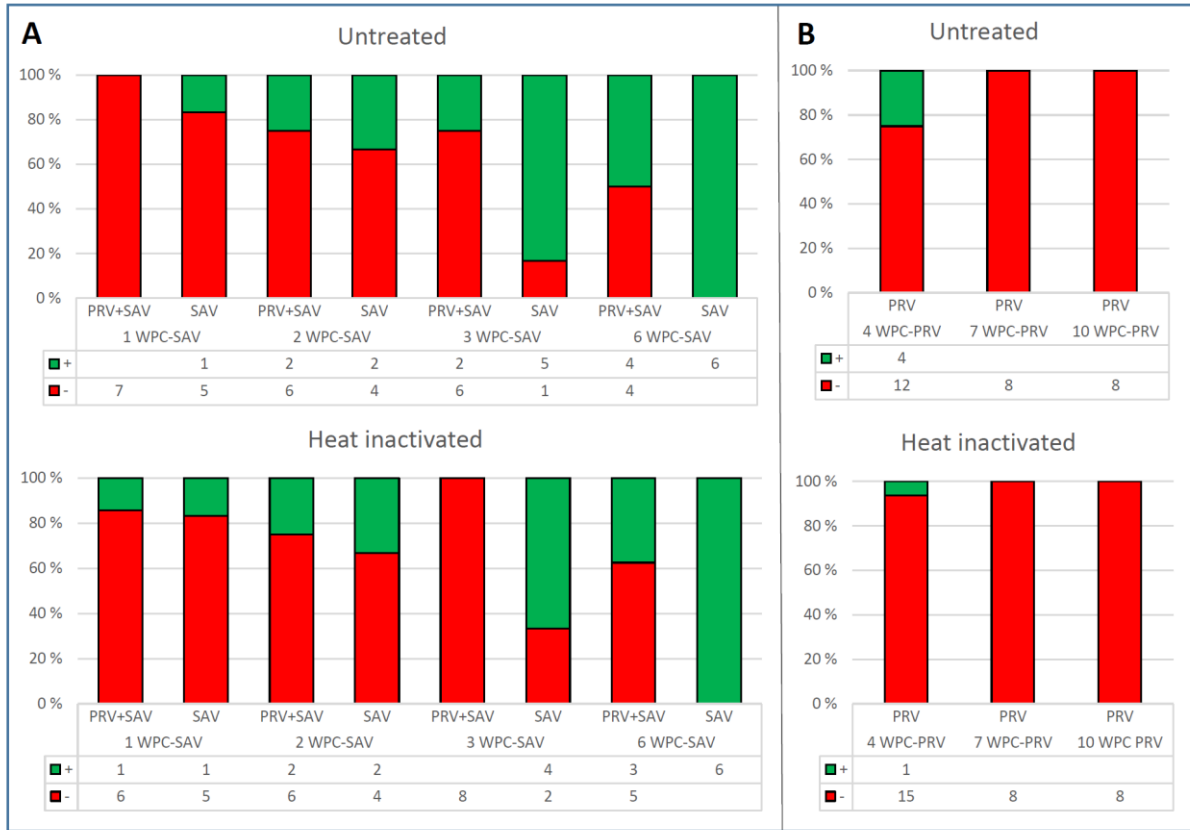
Figure 6. *SAV neutralizing test of plasma samples. Threshold for positive SAV neutralization titer was set to 1:20. Positive samples are indicated with + (green) and not detected by - (red). Number of positive and negative samples is shown in the table below the bar graph. Complement was inactivated by heat treatment (48°C for 20 minutes). A: SAV controls, PRV-SAV co-infected (SAV- early) fish at 1, 2, 3 and 6 weeks post SAV infection. B: PRV infected control fish from 4, 7 and 10 WPC-PRV infection.*

Field samples
The field studies confirmed a high prevalence of PRV after sea transfer, with 96.3% and 100% in the Screening and Longitudinal studies, respectively.

For dead and moribund fish in the Screening data, the correlation between SAV and PRV Ct values was assessed by Spearman's rank correlation. Here, a weak positive correlation ($r = 0.23$, $p < 0.05$) (N = 307) was identified (Figure 7A). However, the scatter plot for each time

point from the Screening data showed large differences in the correlations between SAV and PRV Ct-values (Figure 7B).

In the Longitudinal study, i.e. the natural PD outbreak, the cumulative mortality was approximate 3 % in the two study pens in the study period from the time of the first detection of SAV to the last sampling 11 weeks later. Randomly selected, apparently healthy fish sampled through the PD outbreak period displayed an overall negative correlation ($r = -0.22$, $p < 0.05$) (N = 159) between PRV and SAV Ct values (Figure 8A). From the individual sampling points a similar trend was observed, however no significant correlation was found (Figure 8B). The scatter plot show that there is a large individual variation, and therefor limited explanatory value of PRV on SAV Ct- values.
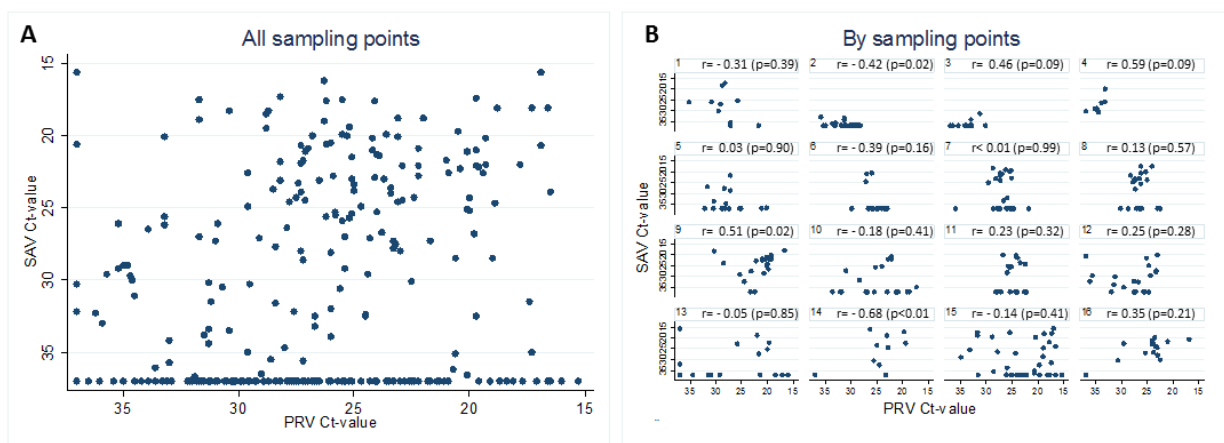

Figure 7. *Screening study: SAV and PRV Ct-values in dead and moribund fish. **A**: Scatterplot from all sampling points, Spearman rank correlation 0.23 (p<0.05). **B**: Scatterplots and Spearman rank correlations by each sampling point.*
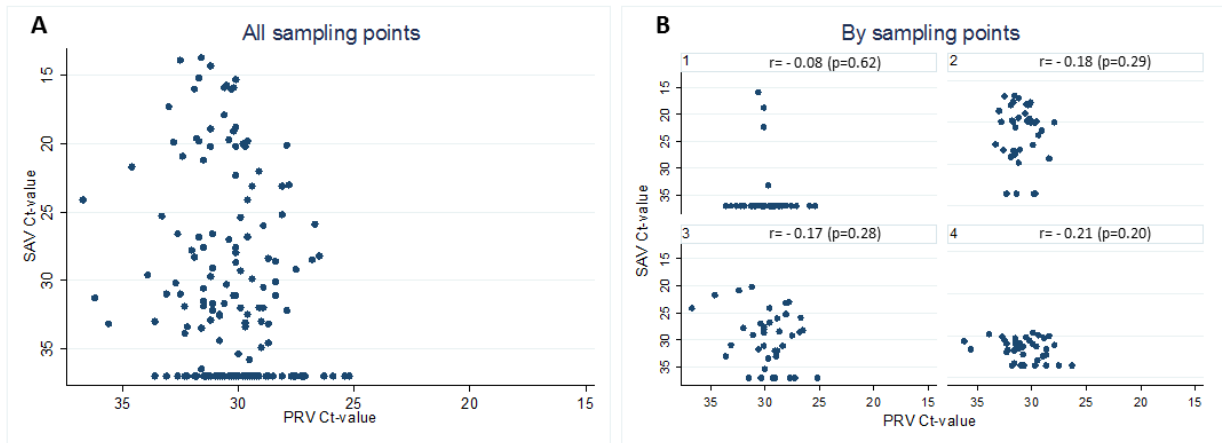
Figure 8. *Longitudinal study: SAV and PRV Ct-values in randomly sampled fish. **A:** Scatterplot for all sampling points Spearman rank correlation -0.22 (p<0.05). **B:** Scatterplots and Spearman rank correlations by each sampling point.*

Discussion

In an earlier study it was found that a primary PRV infection protects against a secondary SAV infection, observed as a reduction in SAV RNA levels in heart and blood and pancreas disease specific lesions, evaluated by an ordinal scoring system in pancreas and heart [18]. However, the mechanism behind this protection is unknown. In the current study, we found that a PRV infection induced and upregulated a broad specterum of innate antiviral immune genes, which may play a protective role against SAV infection. In addition, field data from apparently healthy co-infected fish showed, in line with the experimental trial, a negative correlation between SAV and PRV RNA levels.

The innate antiviral immune response, induced by a virus such as PRV, is mediated through receptor recognition of pathogen associated molecular patterns (PAMPs) such as dsRNA. This triggers responses that activate various signaling pathways, type I IFN being the main pathway, and induces the expression or stimulates a wide range of antiviral effector molecules [42–45]. In this study, we focused on a panel of innate antiviral immune genes where the products interact with different stages of the SAV replication cycle. CH25H is a protein which in mice cells inhibits viral entry of enveloped viruses by blocking membrane fusion [46]. Increased expression of the cytoplasmic dsRNA receptor RIG-I may indicate reinforced awareness to dsRNA. Mx has broad antiviral activity, Mx-transfected fish cells are resistant to SAV infection [47]. PKR inhibits viral mRNA translation through phosphorylation of eIF-2α [48]. ISG-15 is a ubiquitin-like modifier highly upregulated in the innate signaling cascade [49] and bind to both host and viral protein. Binding to capsid protein and thereby interfering with the assembly of the capsid is one of the suggested antiviral mechanism [49]. Viperin inhibits viral replication by interfering with viral budding of enveloped viruses [50]. However, it is induced by a broad specter of non-viral agents as well, indicating a broader functional range [51]. USP18 is a negatively regulated protein of the innate immune system signaling cascade with function of proteolytic activity against ISG-15 [52].

Increased expression of the selected antiviral innate immune genes was demonstrated in blood cells, spleen and heart following PRV infection and lasted for at least ten weeks after cohabitation. In general, the level of innate immune gene expression correlated strongly with the Ct of PRV RNA (Table 2). This is in accordance with previous studies and confirms a strong innate immune response after a PRV infection in Atlantic salmon [30–32]. However, in Sockeye salmon only minor gene regulation was found after PRV infection at the time of the subsequent IHNV infection and no alterations in the secondary infection was observed [19].

This supports the hypothesis that activation of the innate antiviral immune response is central for the observed protection in our co-infection study.

Erythrocytes are the main target cells for PRV and during the peak phase of infection more than 50% are infected [4]. We found that the PRV infection in blood cells interferes with the expression of commonly used reference genes such as EF1α and β-actin, indicating that the viral infection impacts basic cellular processes as EF1α is essential for translation initiation and β-actin is a pivotal part of the endoskeleton [53,54]. Earlier observations have indicated that PRV infection of erythrocytes causes a down-regulation of a vast number of non-immune related genes in blood [30]. Blood cells are present in samples from any organ, i.e. it is intrinsic to an experimental infection with PRV that the expression profile of blood cells will influence the observed expression pattern in any blood containing organ sample. For instance, we found that the expression profile of a blood rich organ such as spleen was more similar to that of blood than compared to that of the heart. Another possible explanation for the observed interference of the reference genes, is changed cellular composition of blood due to the PRV infection. Thus, the expression of EF1α and β-actin were not invariant and could not be used for normalization of gene expression in neither spleen nor blood. However, EF1α was deemed suitable for heart tissue samples and has also previously been used for normalization of PRV RNA from heart [32]. For blood and spleen samples an equal amount of RNA was used for each RTqPCR set-up, and the gene expression levels standardizing to the amount of RNA.

Viral infections will generally interfere with cellular protein expression. Infection with mammalian reovirus (MRV) results in translation inhibition of cellular mRNAs [55] while viral RNA translation is maintained [56]. The inhibition of translation occurs through PKR, which is activated through stimulation by IFN or directly by dsRNA, leading to phosphorylation of the cellular translation initiation factor EIF2α and thus blocking translation [57,58]. Changes in cellular gene regulation could be a secondary effect to the virus-induced translational blocking. The mechanisms by how reoviruses escape cellular translation inhibition is not known in detail. However, it has been suggested that the dsRNA binding viral protein σ3 is central in this regard, inhibiting activation of PKR. The PRV σ3 protein also binds dsRNA in a sequence independent way [59].

Antimicrobial peptides (AMP) are upregulated during viral infections in fish (reviewed in [60]). β-defensins and hepcidins are mainly expressed in mucosal organs, but they are also present in head-kidney, spleen and liver. However, isoform type, the developmental stages of

the fish, species differences and external stimuli all affect the expression patterns of AMPs [60]. In our study, we found no unison regulation of β-defensin and hepcidin 4 WPC-PRV and 10 WPC-PRV in blood, spleen and heart after PRV infection. The only significant upregulation observed was in blood 4 WPC-PRV. Chiou and co-workers (2007) detected increased expression of hepcidin in IHNV infected cells *in vitro* after 12 hours, but it fell below baseline levels 24 hours after infection [61]. The isotype induced and the chosen time-points in our study may have influenced why we did not observe any regulation.

Virus neutralization by the humoral response is an important host mechanism blocking viral attachment and entry into the cell. Neutralizing activity has traditionally been assigned to the production of specific antibodies, but can also be caused by various other components such as innate antibodies and defensins [62]. SAV infection induces neutralizing antibodies that can be assessed in the diagnosis of pancreas disease [41]. In our study, the neutralizing effect of the plasma from the co-infected fish was lower both in titer and in number of positive individuals when compared to the SAV control group. This suggests a less specific humoral response towards SAV in the co-infected fish. Some individuals in the co-infected groups were negative for SAV RNA in the spleen. The low viral load, and hence the reduced SAV antigen exposure in the co-infected individuals, is a likely explanation for the low neutralization activity. The plasma from four PRV infected fish showed SAV neutralization prior to SAV infection at 4 WPC-PRV. Heat treatment removed this effect in three of the four samples. No cross-neutralizing effect was detected in plasma collected 7 and 10 WPC-PRV in the group infected by PRV only. The results indicate that non-specific factors of plasma can neutralize SAV.

The two data sets from field was included to investigate if the two viruses interact with each other during farming conditions. To our knowledge there has only been one previous publication including PRV and SAV co-infection sampled in farms in Norway. The study did not detect any relation between PRV and SAV RNA levels[8].

In the Longitudinal study, HSMI was diagnosed approximate five months before SAV was detected thus indicating that the population were PRV infected prior to SAV exposure, and indeed the PRV prevalence was found to be 100%. However, the exact onset of the PRV infection in the Longitudinal study is unknown. We detected an overall weak negative correlation (r=-0.22 p<0.05) between SAV and PRV Ct values in the randomly sampled, apparently healthy fish. Time after primary infection and the magnitude and duration of the innate antiviral immune response are likely important for the magnitude of the interference

with the secondary infection. Therefore, it is surprising to find negative correlation between SAV and PRV seven to ten months after HSMI was diagnosed. However, recurrent infection with PRV, or infection with an unknown non-virulent virus, cannot be excluded. A recent field study showed a strong negative correlation of viral RNA loads (r = -0.70, p < 0.007) of PMCV and SAV (N=13) but no correlation between PRV and SAV [8], however timing and sequence of the infections were unknown.

A weak positive correlation (r= 0.23 p<0.05) between SAV and PRV was found in dead and moribund fish in the Screening data. However, there was a large variation between individual fish as well as for each sampling point, and again the timing and sequence of the infections were unknown.

Although, one should be aware of that correlation is not necessary causation and field observations should be interpreted with care. The difference in correlation between apparently healthy fish and dead or moribund fish, suggest that health status is of importance for how a primary PRV infection interact with a secondary SAV infection. Further analysis of the field data was abandon due to limited explanatory value of PRV on SAV Ct, demonstrated by the large individual variation, as displayed by the scatter plots (Figure 7 and 8). The combined results of the challenge trial and the field data indicate that time after infection, host and environmental factors play important roles for the outcome.

In conclusion, the expression of innate antiviral immune genes after a PRV infection was consistent with protection against a secondary SAV infection. The field data of co-infected apparently healthy fish and moribund or dead fish suggest that health status may alter the interaction.

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