

Molecular studies of
***Piscine orthoreovirus* proteins**

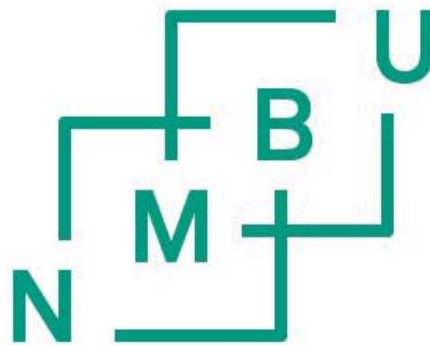
Viral factories, kinetics and vaccination

Philosophiae Doctor (PhD) Thesis

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Viruses, not lions, tigers or bears, sit masterfully above us on the food chain of life, occupying a role as alpha predators who prey on everything and are preyed upon by nothing

Claus Wilke and Sara Sawyer, 2016

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Oslo, 2017

Hanne Merethe Haatveit

Abbreviations

ARV	Avian orthoreovirus
AqRV	Aquareovirus
BroV	Broom orthoreovirus
BRV	Baboon orthoreovirus
CHSE	Chinook salmon embryo cells
CNS	Central nervous system
CMS	Cardiomyopathy syndrome
CMV	Cytomegalovirus
CPE	Cytopathic effect
Ct	Cycle threshold
dsRNA	Double-stranded RNA
DTT	Dithiothritol
EF1 α	Elongation factor 1 α
EIBS	Erythrocytic inclusion body syndrome
eIF2	Eukaryotic initiator factor 2
EPC	Epithelioma Papulosum Cyprini
ER	Endoplasmic reticulum
FAST-protein	Fusion associated small transmembrane protein
GCRV	Grass carp reovirus
GI	Gastrointestinal tract
HSMI	Heart and skeletal muscle inflammation
ICTV	International Committee on Taxonomy of Viruses
IHC	Immunohistochemistry
IHN	Infectious hematopoietic necrosis
IFN	Interferon
i.p.	Intraperitoneal
IPN	Infectious pancreatic necrosis
IRF	Interferon regulatory factors
ISA	Infectious salmon anemia
ISG	IFN-stimulated gene
ISVP	Infectious subviral particle
JAK-STAT	Janus kinase-Signal transducers and activators of transcription
JAM-A	Junction adhesion molecule A
KDa	KiloDalton
NBV	Nelson Bay orthoreovirus
NF $\kappa\beta$	Nuclear factor $\kappa\beta$
NK	Natural killer
NS	Non-structural

MAB	Monoclonal antibody
MDA	Melanoma differentiation-associated gene
MHC	Major histocompatibility complex
MRV	Mammalian orthoreovirus
mRNA	Messenger RNA
MT	Microtubule
ORV	Orthoreovirus
ORF	Open reading frame
PAb	Polyclonal antibody
PABP	Poly (A) binding protein
PAMPs	Pathogen associated molecular patterns
PD	Pancreas disease
p.i.	Post infection
PKR	dsRNA-activated protein kinase
PKZ	Z-binding protein kinase
PRV	Piscine orthoreovirus
RdRp	RNA-dependent-RNA-polymerase
RIG-I	Retinoic acid-inducible gene I
RNase L	Ribonuclease L (latent)
Reo	Respiratory enteric orphan
RRV	Reptilian orthoreovirus
RT-qPCR	Real-time quantitative polymerase chain reaction
SAV	Salmonid alphavirus
SDS	Sodium dodecyl sulfate
T1L	Type 1 Lang
T3D	Type 3 Dearing
TEM	Transmission electron microscopy
TLR	Toll-like receptor
TTA	Tetradecylthioacetic acid
USP	Ubiquitin specific peptidase
VF	Viral factory
VLP	Virus-like particle
wpc	Weeks post challenge

List of papers

Paper I

The non-structural protein μ NS of *Piscine orthoreovirus* (PRV) forms viral factory-like structures

Authors: Haatveit HM, Nyman IB, Markussen T, Wessel Ø, Dahle MK, Rimstad E.

Published: *Veterinary Research* 2016, 47:5

Paper II

Viral protein kinetics of *Piscine orthoreovirus* infection in Atlantic salmon blood cells

Authors: Haatveit HM, Wessel Ø, Markussen T, Lund M, Thiede B, Nyman IB, Braaen S, Dahle MK, Rimstad E.

Published: *Viruses* 2017, 9, 49

Paper III

DNA vaccine expressing the non-structural proteins of *Piscine orthoreovirus* induces moderate protection against heart and skeletal muscle inflammation in Atlantic salmon (*Salmo salar*)

Authors: Haatveit HM, Hodneland K, Braaen S, Hansen EF, Frost P, Rimstad E.

Manuscript

Summary

Piscine orthoreovirus (PRV) is ubiquitous in farmed Atlantic salmon (*Salmo salar*) and the cause of heart and skeletal muscle inflammation (HSMI). PRV is an orthoreovirus in the family *Reoviridae* and shows several similarities to the *Mammalian orthoreovirus* (MRV).

Reovirus non-structural proteins are involved in the establishment of viral factories, and viral factory-like structures have been observed in PRV infected Atlantic salmon erythrocytes. By fluorescent microscopy, dense globular inclusions resembling viral factories, formed by the non-structural protein μ NS of PRV, were detected in transfected fish cells. In co-transfection experiments with μ NS, the σ NS, μ 2 and λ 1 proteins were recruited to the globular structures. Expression of the N-terminal 401 amino acids of the μ NS protein did not induce viral factory-like structures, mapping this feature to the remaining C-terminal 351 amino acids.

By means of a cohabitant challenge lasting for 8 weeks, the kinetics of viral RNA, viral protein and antiviral immune response in blood cells from PRV-infected Atlantic salmon were investigated. The study showed that PRV infection has an acute phase in blood cells with high virus production and innate antiviral gene expression before the infection subsides to a low persistent level. Different size variants of the μ NS and the outer capsid protein μ 1 were observed at specific time points during infection. Interestingly, a proteolytic cleavage fragment of the μ 1 protein was the only viral protein detectable 7 weeks post challenge, indicating that this fragment may be involved in the mechanisms of persistent infection.

At last, two experimental challenges testing different DNA vaccines against HSMI were performed. The results showed that a vaccine expressing the viral factory forming non-structural proteins μ NS and σ NS, in combination with the structural outer capsid protein σ 1, induced moderate protection against HSMI in Atlantic salmon.

To conclude, our studies have shown that μ NS is the main protein involved in viral factory formation during the acute phase of PRV infection and the protein, in combination with σ NS and σ 1, also has the ability to induce protection against HSMI when delivered as a DNA vaccine.

Sammendrag (Summary in Norwegian)

Piscine orthoreovirus (PRV) er ubikvitær hos oppdrettslaks og årsaken til hjerte- og skjelettmuskelbetennelse (HSMB). PRV er et orthoreovirus i familien *Reoviridae*, og har flere likhetstrekk med *Mammalian orthoreovirus* (MRV).

Ikke-strukturelle proteiner fra reovirus er involvert i etablering av virusfabrikker ved infeksjon, og virusfabrikk-liknende strukturer er tidligere observert i PRV infiserte erythrocytter fra laks. Fluorescensmikroskopi viste virusfabrikk-liknende globulære inklusjoner dannet av det ikke-strukturelle proteinet μ NS i transfekterte fiskeceller. I ett ko-transfeksjonsforsøk med μ NS ble σ NS, μ 2 og λ 1-proteiner rekruttert til de globulære strukturene. Det ble ikke observert virusfabrikk-liknende strukturer når kun den N-terminale delen av μ NS ble uttrykt, noe som knytter denne egenskapen til de gjenværende 351 C-terminale aminosyrene.

Prøvemateriale fra et 8-ukers kohabitant smitteforsøk ble benyttet til å studere viruskinetikk, proteinkinettikk og antiviral immunrespons i blodceller fra PRV-infisert laks. Studien viste at PRV gir en akutt, massiv infeksjon i blodceller som kun strekker seg over noen få uker før fiskens immunrespons dirigerer infeksjonen over i en persistent fase. Ulike størrelsesvarianter av μ NS og det ytre kapsidprotein μ 1 ble observert ved bestemte tidspunkter under infeksjon. Ett proteolytisk kløyvingsfragment av μ 1-proteinet var det eneste påvisbare virusprotein 7 uker etter smitte, noe som indikerer at dette fragmentet kan være involvert i mekanismer som fører til persistent infeksjon.

Til slutt ble det utført to smitteforsøk hvor ulike DNA-vaksiner mot HSMB ble testet. Resultatene viste at en vaksine som uttrykte de virusfabrikk-dannende proteinene μ NS og σ NS, i kombinasjon med det strukturelle ytre kapsid proteinet σ 1, induerte moderat beskyttelse mot HSMB i Atlantisk laks.

Våre studier har vist at μ NS er hovedprotein involvert i dannelsen av virusfabrikker og at PRV gir en akutt infeksjon av relativt kort varighet før infeksjonen går over i en persistent fase. Kombinasjonen av μ NS og σ 1, har evnen til å induere moderat beskyttelse mot HSMB når de uttrykkes i en DNA-vaksine.

1. Introduction

1.1. Background

Viral diseases pose a significant threat to the productivity of aquaculture and thus to its future in food production. Aquaculture is among the fastest-growing food production industries globally, and is expected to increase further to meet future food demand. With food fish supply increasing at an average annual rate of 3.2%, global aquaculture production is estimated to provide 62% of all human fish food by 2030 [1]. In 2015, ambitions for a fivefold increase in the Norwegian aquaculture industry were announced at political level.

Atlantic salmon (*Salmo salar*) is farmed in Norway, Chile, Scotland, Canada, Ireland, Faeroe Islands, Iceland, USA and Australia, with Norway as the dominant producer [1]. The environment encountered by Atlantic salmon in aquaculture is markedly different from that of wild fish. Farming has introduced reduced environmental variation, high fish densities, excess food availability, absence of predation, no competition for mates and strong selection for traits such as rapid growth and delayed sexual maturation [2]. Genetic and morphological changes in response to artificial selection and genetic drift within a limited population size, may lead to reduced fitness compared to wild individuals. In addition, fish kept at high stocking density with frequent handling such as de-lousing and sorting, experience a stressful environment [3]. An intrinsic property of large scale farming is facilitated spread of pathogens. Today, one of the main challenges in aquaculture industry is viral diseases (Table 1) [3].

Table 1. Incidence of viral diseases in farmed salmonids in Norway from 2006 – 2016.

Figures for notifiable and non-notifiable diseases are based on data from samples tested by the Norwegian Veterinary Institute. Modified from Fish Health Report 2016, the Norwegian Veterinary Institute 2017 [4].

	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
ISA	4	7	17	10	7	1	2	10	10	15	12
PD	58	98	108	75	88	89	137	99	142	137	138
HSMI	94	162	144	139	131	162	142	134	181	135	101
IPN	207	165	158	223	198	154	119	56	48	30	27
CMS	80	68	66	62	49	74	89	100	107	105	90

Piscine orthoreovirus (PRV) is a ubiquitous virus in the marine phase of farmed Atlantic salmon and the cause of heart and skeletal muscle inflammation (HSMI) [5]. HSMI has emerged from its discovery in 1999 to 130-150 annual outbreaks the last years (Table 1). The cumulative mortality varies from negligible to 20%, while the morbidity is almost 100% in affected cages [6]. The condition leads to significant economic loss in Atlantic salmon aquaculture in Norway. PRV is also associated with the occurrence of melanised foci in the white musculature of Atlantic salmon [7].

Characterization of the causative agent, including virulence factors and immune responses of the host, modes of transmission and knowledge of survival of its infectivity outside the host, are crucial for understanding and preventing the spread of PRV and possible disease outcomes.

1.2. *Piscine orthoreovirus*

PRV was identified in 2010 after high-throughput pyrosequencing of serum from HSMI affected fish. The viral genome resembled that of reoviruses and PRV was tentatively assigned to the family *Reoviridae*, genus *Orthoreovirus* [8, 9]. International Committee on Taxonomy of Viruses (ICTV) approved this assignment and the name of the virus in 2015. Orthoreoviruses are ubiquitous in various animal species, but originally only found to be of pathogenic significance in farmed poultry and recently in farmed fish [10, 11]. Recently, variants of mammalian orthoreoviruses (MRVs) has been found to trigger inflammatory responses that may lead to the development of celiac disease [12], indicating that assumed non-pathogenic reovirus may have long-term effects not observed at the acute infection. PRV is an *orthoreovirus* that infects and replicates at low temperatures. The virus has been detected in both farmed and wild Atlantic salmon, but no lesions consistent with HSMI have been found in wild populations [13].

1.2.1. Taxonomy

Family *Reoviridae*

The *Reoviridae* family is a large and diverse group of spherical non-enveloped viruses containing 9 - 12 segments of linear dsRNA [14]. Reovirus was first classified in 1959 as a group of viruses typically isolated from human respiratory and gastrointestinal tract, often without any association to disease (reo = respiratory, enteric, orphan) [15]. In the 1970ies, the *Reoviridae* family was arranged into several genera and it has expanded ever since. Today, the family comprises 15 genera with a broad host range from plants, insects, to fish, reptiles, bird and mammals. The genera is divided into two subfamilies, *Spinareovirinae* and *Sedoreovirinae*. Grouping to the subfamilies is based upon the presence or absence of turret structures at the icosahedral vertices of the virion.

Genus *Orthoreovirus*

Orthoreoviruses belongs to the subfamily *Spinareovirinae*, the “turreted” group of reoviruses. There are six approved species groups of orthoreoviruses (Table 2): *Mammalian orthoreovirus* (MRV), *Avian orthoreovirus* (ARV), *Baboon orthoreovirus* (BRV), *Nelson Bay orthoreovirus* (NBV), *Reptilian orthoreovirus* (RRV) and *Piscine orthoreovirus* (PRV). Additionally, *Broome orthoreovirus* (BroV), isolated from fruit bat, represents a new, though not formally recognized orthoreovirus [16].

Table 2. Taxonomic placement of *Piscine orthoreovirus*.

Family	Subfamily	Genus	Species
<i>Reoviridae</i>	→ <i>Spinareovirinae</i>	→ <i>Orthoreovirus</i>	→ <i>Mammalian orthoreovirus</i>
		<i>Aquareovirus</i>	<i>Avian orthoreovirus</i>
		<i>Oryzavirus</i>	<i>Baboon orthoreovirus</i>
	<i>Sedoreovirinae</i>	<i>Fijivirus</i>	<i>Nelson bay orthoreovirus</i>
		<i>Mycoreovirus</i>	<i>Reptilian orthoreovirus</i>
		<i>Cypovirus</i>	<i>Piscine orthoreovirus</i>
		<i>Idnoreovirus</i>	
		<i>Dinovrnavirus</i>	
		<i>Coltivirus</i>	

Orthoreoviruses contain 10 dsRNA genomic segments distributed in groups of three large (L1, L2, L3), three medium (M1, M2, M3) and four small (S1, S2, S3, S4) segments. The genome encodes at least 12 proteins mostly denoted after the corresponding greek letter (λ , μ and σ) from the encoding gene segment (L, M and S) [15].

The orthoreoviruses are functionally divided into two general subgroups, the fusogenic and non-fusogenic reoviruses, based on the ability of the virus to induce cell-cell fusion and syncytium formation in infected cells [17]. This property is mediated by the fusion-associated small transmembrane (FAST) protein and can be observed as multinucleated syncytia in culture [18]. MRV and PRV are currently the only non-fusogenic orthoreoviruses [17, 19].

MRV is the prototype species and has been studied extensively as a model system for virus entry, replication and pathogenesis [20-22]. Reoviruses are known for their oncolytic properties and the type 3 Dearing (T3D) strain of MRV is one of the therapeutic oncolytic viruses in clinical development [23]. MRV has been isolated from several mammalian species, but is rarely associated with clinical disease in humans [20, 21]. However, in mice, MRV may cause a number of disease conditions involving various organs like the central nervous system (CNS), intestine and heart [24, 25].

ARV has been isolated from chickens, ducks, geese, turkeys and psittaciformes, and is associated with a number of different disease states, including arthritis and tenosynovitis, enteric and respiratory diseases, hepatitis and malabsorption, in poultry [26-28]. BRV, NBR and RRV has also been associated with disease in baboons, humans and reptilians, respectively [29-32].

PRV causes HSMI, is associated with melanised foci in Atlantic salmon [7, 8], and is the only species within this genus that infects fish. Recently, a variant of PRV was also demonstrated to be the etiologic agent of erythrocytic inclusion body syndrome (EIBS), a condition associated with anemia and mass mortality in juvenile Coho salmon (*Onchorhynchus kisutchi*) [33]. In addition, yet another PRV variant is associated with anemia and an HSMI-like disease in rainbow trout [34]. Orthoreoviruses have been found in non-salmonid fish, but these viruses are not yet assigned to any virus species [35].

1.2.2. Genome and proteins

PRV is a spherical, non-enveloped virus with an outer diameter of 70 nm and an electron-dense center of 30 nm representing the centrally condensed RNA genome[5].

The genome of PRV has a total size of 23 320 nt [8, 36]. Currently, the PRV genome is predicted to encode at least 12 primary translation products where putative functions are assigned (Table 3). However, the functions and properties of the different proteins are only partly described [9, 37]. Based upon sequence homology to MRV, ARV and *Grass carp reovirus* (GCRV, genus *Aquareovirus*) and the presence of conserved structures and motifs, nine of the deduced translation products are assumed to be structural components forming an inner core and an outer capsid of the viral particle, while three of the translation products are considered non-structural proteins [9].

Table 3. Putative location and function of PRV proteins.

List of PRV encoded proteins and their predicted location and function based on comparison with the properties of MRV, ARV and GCRV. Modified from Markussen et al, PLoS One 2013 [9].

Segment	Protein	Length (aa)	Weight (kDa)	Putative function
L1	λ 3	1286	144.5	RNA-dependent RNA polymerase
L2	λ 2	1290	143.7	Guanylyltransferase, methyltransferase
	p11			Hypothetical protein
L3	λ 1	1282	141.5	Helicase, NTPase, RNA triphosphatase
M1	μ 2	760	86.0	NTPase, RNA triphosphatase, RNA binding
M2	μ 1	687	74.2	Outer capsid protein, membrane penetration
	μ 1C		69.8	
M3	μ NS	752	83.5	Non-structural protein (inclusion formation)
S1	σ 3	330	37.0	Outer capsid protein, zinc metalloprotein
	p13	124	13.0	Cytotoxic, integral membrane protein
S2	σ 2	420	45.9	Inner capsid protein, RNA binding
	p8			Hypothetical protein
S3	σ NS	354	39.1	Non-structural protein (inclusion formation)
S4	σ 1	315	34.6	Cell attachment protein

Inner capsid proteins

In orthoreoviruses, two proteins, $\lambda 1$ and $\sigma 2$, form the inner core, with two additional proteins, $\lambda 3$ and $\mu 2$, present in small amounts (Figure 1) [21]. MRV $\lambda 1$ is the major core protein forming an icosahedral shell stabilized by the $\sigma 2$ protein, the two-dimensional structures are conserved in PRV $\lambda 1$ and $\sigma 2$, despite low sequence homology to MRV [9, 38].

Outer capsid proteins

The mammalian orthoreovirus core is coated with a well-defined outer capsid composed of hexagonal and pentagonal subunits formed by the $\mu 1$, $\sigma 1$ and $\sigma 3$ proteins, with pentamers of the $\lambda 2$ protein forming the turrets at the 12 vertices of the core (Figure 1) [21, 38]. A trimer of $\sigma 1$ is associated with each $\lambda 2$ turret, making up the spikes, and known to be the cell attachment protein and serotype determinant for MRV [39-43]. Analysis of PRV gene segment S4 indicating the presence of α -helical coiled-coil structure(s) in the N-terminal region, shows that PRV $\sigma 1$ most likely is the cell attachment protein corresponding to those of other orthoreoviruses [9].

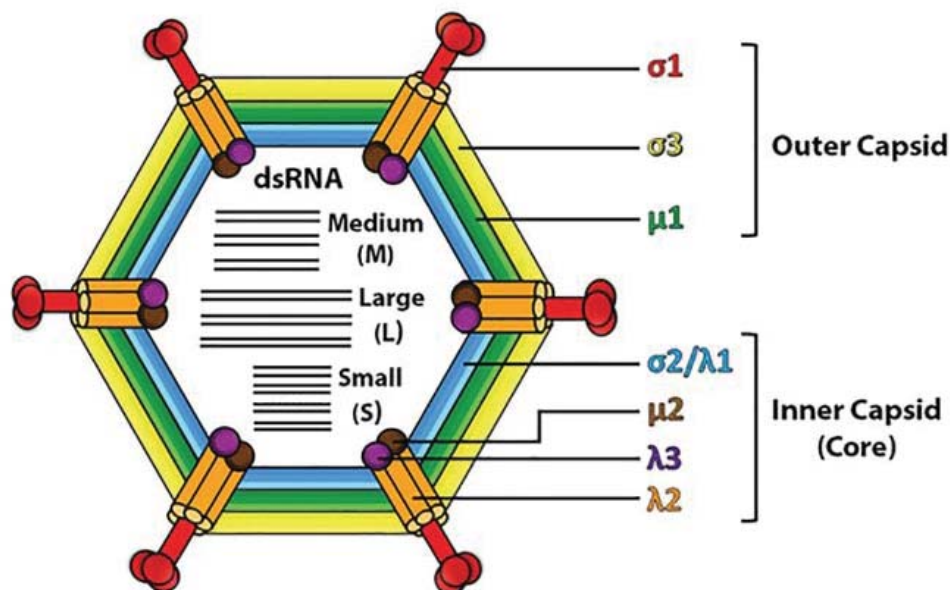


Figure 1. The orthoreovirus particle

The model illustrates inner and outer capsid proteins and the dsRNA genome of orthoreoviruses. Modified from Mohamed et al, Viruses 2015 [44].

The MRV $\mu 1$ and $\sigma 3$ proteins constitute the majority of the outer capsid, coating the core as a heterohexamer [45]. In the virion outer capsid, most of the $\mu 1$ protein exists in the form of 6

$\mu 1C$ and $\mu 1N$ fragments generated by proteolytic cleavage [46-49]. The $\mu 1N$ fragment is myristoylated and important for membrane penetration [50, 51]. Both the cleavage and myristolization sites in MRV $\mu 1$ are conserved in the corresponding PRV protein [9, 19]. Cytoplasmic $\sigma 3$ protein binds dsRNA in a sequence independent manner which inhibits the interferon-induced, dsRNA activated kinase (PKR) and/or the RNase L system, and in turn prevents translational shutoff [52-54]. PRV $\sigma 3$ is shown to have important zinc-finger motifs and dsRNA binding properties, although no specific domain is shown to be responsible for dsRNA binding [9, 37].

Non-structural proteins

Non-structural (NS) proteins are proteins encoded by the viral RNA, but not part of the viral particle itself. The non-structural proteins are only produced in the cell to serve a role in replication.

PRV gene segment S1 is bicistronic and has an internal open reading frame (ORF) encoding a 124 aa (13 kDa) cytotoxic protein, p13 [9]. The p13 protein binds to intracellular membranes and co-localizes with a marker for the trans-Golgi network [19]. It does not co-localize with the $\sigma 3$ protein, also encoded by the S1 segment [55]. In addition, both S2 and L2 are putative polycistronic gene segments, however, neither the presence of the gene products, nor functional properties of these, have been investigated [9].

PRV gene segments M3 and S3 encodes the non-structural proteins μNS and σNS , respectively [9]. PRV μNS is homologous to MRV and ARV μNS , both encoded by gene segment M3. Similarly, PRV σNS is the homologue to MRV and ARV σNS , encoded by gene segment S3 and S4, respectively [9]. There is very low amino acid sequence identity (17 – 20 %) for both the μNS and σNS proteins between MRV, ARV and PRV, however some assumed functional motifs are conserved [9].

For both MRV and ARV M3, two translational products have been reported where μNS represents the full-length isoform [56]. PRV μNS consists of 752 amino acids (aa) with a theoretical weight of 83,5 kDa, equivalent to the full-length MRV homologue [9]. PRV M3 has only one ORF coherent with the Kozak rule of transcriptional activation [55]. For σNS , the similarities between PRV, MRV and ARV is slightly higher than for μNS . All three

homologous gene segments contain one single ORF and the protein size matches closely at about 39 kDa [9].

A common feature of the non-structural proteins of reoviruses is their ability to form viral factories (VFs), which facilitates successful replication [57, 58]. This feature has been studied extensively, but the mechanisms by which a viral factory forms and supports viral replication are still not completely elucidated [59]. Similar viral factories are observed during PRV infection [60, 61].

1.2.3. Target cells

Currently, no cellular receptor for PRV has been found. For MRV, the $\sigma 1$ protein is shown to serve as a viral receptor recognition protein and distinct domains of the protein may be involved in binding different types of receptors on discrete populations of target cells [41, 62]. The MRV $\sigma 1$ protein contains a domain that binds to sialic acids, and another domain, which binds the receptor junctional adhesion-molecule-A (JAM-A). Sialic acids are widely distributed on cell surfaces, and MRV's binding to these is thought to help the virus get into closer contact with the proper receptor. Several of the amino acid residues in MRV $\sigma 1$ involved in sialic acid binding are conserved in PRV [55]. MRV has a tropism for numerous different cell types including myocytes and neurons [40, 41, 62, 63]. MRV Type 3 (T3) spread from the enteric tract into mononuclear cells, ileal Peyer's patches, neurons of the myenteric plexus adjacent to Peyer's patches and then into the brain stem neurons through the vagus nerve [64]. Reovirus Type 1 Long (T1L) follows a different pathway as it is transported across M cells into Peyer's patches, mesenteric lymph nodes and, ultimately, to the spleen [65].

Erythrocytes are major target cells for PRV and during peak phase of infection over 50 % of these cells can be infected [60]. PRV also infects myocytes of the heart and skeletal muscles [11]. However, the relative amount of PRV RNA in blood is significantly higher than what is found in heart or skeletal muscle during peak phase of experimental infection, while viral RNA load in spleen and head kidney bypasses that of blood in later phases of infection [60].

Piscine erythrocytes

Piscine erythrocytes are primarily produced in the anterior quarter of the kidney and the pulp of the spleen, but also in the renal interstitium in the posterior part of the kidney [66]. Piscine, reptile and avian erythrocytes, opposed to mammalian erythrocytes, are nucleated and contain the transcriptional and translational machinery necessary for expression of mRNA and proteins [67]. The main function is respiratory gas exchange, but it has been speculated if non-mammalian erythrocytes also have other functions [68] and recent studies have indicated a role in pathogen recognition and immune response [67, 69]. Cold-water fish, like Atlantic salmon, have lower metabolism and therefore less oxygen demand. However, erythrocytes are still the most abundant circulatory cell.

Mature salmonid erythrocytes are ellipsoidal, 13 - 16 μM long, 7 - 10 μM broad, and have a centrally located nucleus with compact chromatin (Figure 2) [66, 70, 71]. Immature erythrocytes, called reticulocytes, have a more oval shape with less dense chromatin and more granular substance within the cytoplasm [70, 72]. During maturation of the erythrocytes, there is an increasing loss of cytoplasmic organelles accompanied by augmented hemoglobin content and reduced aerobic metabolic rate [73, 74].

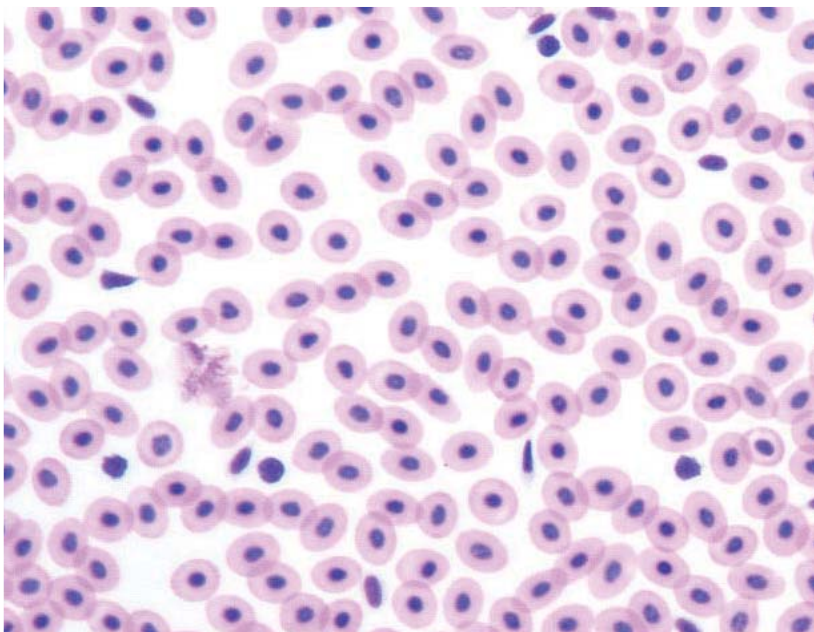


Figure 2. Blood smear from fish showing piscine erythrocytes.

Courtesy of Southern Illinois University at Carbondale, Department of Zoology (www.siumed.edu).

1.2.4. Transmission

Epithelial surfaces such as the gills, skin, basis of fins and intestine are major sites of viral entry in fish species [75]. PRV is believed to transmit horizontally through water and cohabitation with infected fish induce characteristic HSMI lesions in the cohabitants [76, 77]. A study of brood stock fish showed that fertilized eggs produced by PRV positive parents were PRV negative, indicating that vertical transfer is not a major route of transmission [78]. A recent study showed that Atlantic salmon was efficiently infected by PRV after anal intubation, leading to transfer of virus from the intestinal lumen, to blood and subsequently to the heart [79]. The virus infects and replicates in circulating erythrocytes prior to infection of heart myocytes [11, 60, 61]. Further, PRV was also found to shed into faeces, indicating a potential virus transmission route [79].

1.3. *Replication of orthoreoviruses*

Viruses are intracellular parasites, and depend on cell functions for their morphogenesis and propagation [57]. The inability to propagate PRV in laboratory cell lines has significantly delayed the study of viral replication and pathogenesis of HSMI. However, reoviruses in general are well characterized and MRV is commonly used as a model to understand basic mechanisms in reovirus replication.

1.3.1. Receptor-mediated endocytose

Reoviruses infect the target cell through receptor-mediated endocytosis (Figure 3). The MRV $\sigma 1$ protein initially binds with low affinity to carbohydrate structures on the cell surface (the amino acids of $\sigma 1$ involved in this binding is conserved between MRV and PRV). The carbohydrates serve as co-receptors, facilitating the high affinity binding of $\sigma 1$ to the main receptor for MRV, junction adhesion molecule-A (JAM-A) [39-41]. This triggers internalization of the virus into the cell by receptor-mediated endocytosis in clathrin-coated vesicles [80, 81]. Further, the vesicle loses its clathrin-coating, matures into an early endosome and is acidified due to influx of H^+ ions by active pumping [82-85].

In the late endosome, proteolysis of outer capsid proteins activates reovirus particles for the next step in infection. The virions are turned into infectious subviral particles (ISVPs) [86, 87] and further into core particles that enter the cytoplasm [48, 88]. The ISVPs are formed by digestion of the outer capsid protein $\sigma 3$ and proteolytic cleavage of the outer capsid protein $\mu 1$ [89]. Mature virions can also be subject to extracellular proteolysis and formation of ISVPs under certain conditions, like in the intestine.[90, 91]. The ISVPs created in the intestine are highly infectious and may use receptor-independent pathways to enter host cells [92].

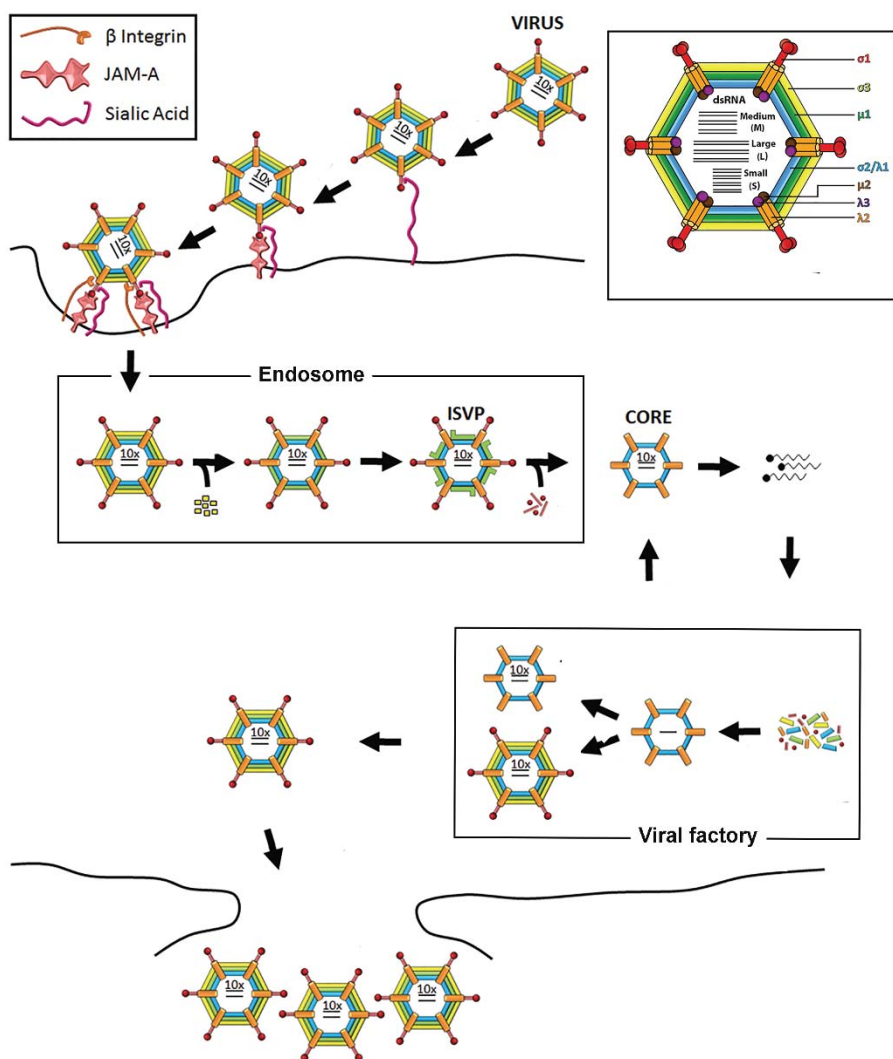


Figure 3. Replication of Mammalian orthoreovirus.

The model illustrates the replication cycle of MRV including cell attachment, endocytosis, uncoating, membrane penetration, transcription, translation, assembly and release of infectious virions. Modified from Mohamed et al, Viruses 2015 [44].

1.3.2. Membrane penetration

As non-enveloped viruses cannot fuse with endosomal membranes for interaction and penetration, other mechanisms must be utilized for the reovirus particles to gain access to the cytoplasm [93]. Conversion of ISVPs to core particles facilitates this process. The ISVPs are thought to be required intermediates during an infection as they are uniquely capable of initiating penetration of a cell membrane to provide access to the cytoplasm (Figure 3) [94]. The $\mu 1$ protein, which covers most of the surface of ISVPs, is processed resulting in release of the membrane penetrating fragment $\mu 1N$ [45, 95]. The $\sigma 1$ protein is released from the particle and the $\mu 1N$ fragment interacts with the endosomal membrane forming size selective pores through which the core particles can pass [96]. Core particles, in contrast to the ISVPs, are not infectious, but highly active mRNA transcription machines [86, 97].

1.3.3. Transcription and translation

In the cytoplasm, the core particles do not further disassemble and the transcription occurs within the core particle. Early transcription is initiated from the dsRNA genome inside the core synthesizing plus-strand RNA transcripts which are released through the $\lambda 2$ turret (Figure 3). Transcription is performed by the $\lambda 3$ RNA-dependent RNA polymerase and transcriptase cofactor $\mu 2$, while capping is mediated by $\lambda 2$, respectively, all enzymes encoded by viral genes and present in the virion [98]. Each of the ten gene segments may represent an independent transcriptional unit and the different transcripts are produced at rates which are approximately proportional to the reciprocal of their length [49, 99-102]. Neither dsRNA nor negative-strand RNA exist in free form in the cytoplasm. The capped, non-polyadenylated plus-strand transcripts can serve both as mRNAs for translation and as templates for minus strand synthesis within progeny particles [98, 103].

In cell cultures infected with MRV, primary transcripts are detected 2 hr after infection, reach a maximum after 6 to 8 hr, and decrease to undetectable levels by 12 hr [49, 104]. Transcripts from gene segment L1, M3, S3 and S4, encoding the $\lambda 3$, μNS , σNS and $\sigma 1$ proteins, respectively, are made preferentially during the first hours of infection [102, 105, 106]. Secondary transcripts are mediated by newly assembled particles and are more effective producers of transcripts during infection [107]. The transcripts exit through the $\lambda 2$ turret

structure and are translated into viral proteins in the cytoplasm by host ribosomes (Figure 3) [108, 109]. Reovirus mRNA lack the 3`polyA tail which is known to bind poly(A) binding protein (PABP) important for stabilization and recruitment of translation factors [103, 110, 111]. How reovirus mRNA is stabilized and translated in the cytoplasm is therefore still not completely elucidated.

Shortly after the onset of infection, there is a gradual increase in synthesis of reovirus proteins. Newly synthesized MRV proteins can be detected already 2 hr post infection and by 10 hr, most of the proteins synthesized in the infected cell are of viral rather than cellular origin [49]. A study performed on aquareovirus where infected cell cultures were harvested at different time points post infection (p.i.) revealed that NS80, the MRV and PRV μ NS equivalent, could be detected at 6 hr p.i. while the structural inner core and outer capsid proteins were detected 9 and 12 hr p.i. [112].

The newly synthesized viral proteins are assembled in structures called viral factories, organized by the non-structural protein μ NS. In the viral factories, replication, packaging of viral genomes, and subsequent assembly of novel viral particles occur [21, 57, 58, 113]. Many of the reovirus structural proteins exhibit some degree of self-assembly in the absence of viral RNA or the complete set of viral proteins. The major inner capsid proteins λ 1 and σ 2 form particles morphologically similar to core shells, and when λ 2 and λ 3 are co-expressed with these, they are incorporated into the particle as well [114]. In the cytoplasm of infected cells, 90 % of free M2-encoded protein is μ 1, while 95 % of the protein complexed with σ 3 has been cleaved to form μ 1C, suggesting that cleavage of μ 1 may be linked to formation of complex with σ 3 [47].

During core assembly, plus strand RNA is encapsulated and minus strand RNA is synthesized in the core to form the dsRNA genome [115]. This mechanism keeps both the dsRNA and negative-sense RNA shielded from cytoplasmic receptors of the innate immune system as neither dsRNA nor negative strand RNA are found free in the cytoplasm [116]. The core cavity of the virion encompasses one single copy of each genomic segment, which each associate with a separate λ 3 polymerase [117].

1.3.4. Viral factories

A common feature of the non-structural proteins of reoviruses is their ability to form viral factories (Figure 4) [57, 118]. Viral factories are intracellular compartments or inclusions where replication, packaging and assembly of novel viral particles occur [57]. Several RNA and DNA viruses that replicate in the cytoplasm form viral factories that require re-localization of organelles, reorganization of cellular membranes, and changes in the distribution and dynamics of the cytoskeleton [113].

Viral factories commonly form as invaginations in organelles such as mitochondria, endoplasmic reticulum (ER), lysosomes, peroxisomes, Golgi apparatus or chloroplasts [59, 119]. Recent studies has associated reovirus inclusions with several of these organelles, i.e. mitochondria, ER elements and a membranous web which may function as a physical scaffold for inclusion formation [59]. Mitochondria may be used as an energy source to power viral replication and also supply host factors required for viral genome synthesis and particle assembly [119]. The virus factory gives space and eases coordination of viral genome replication and assembly [119].

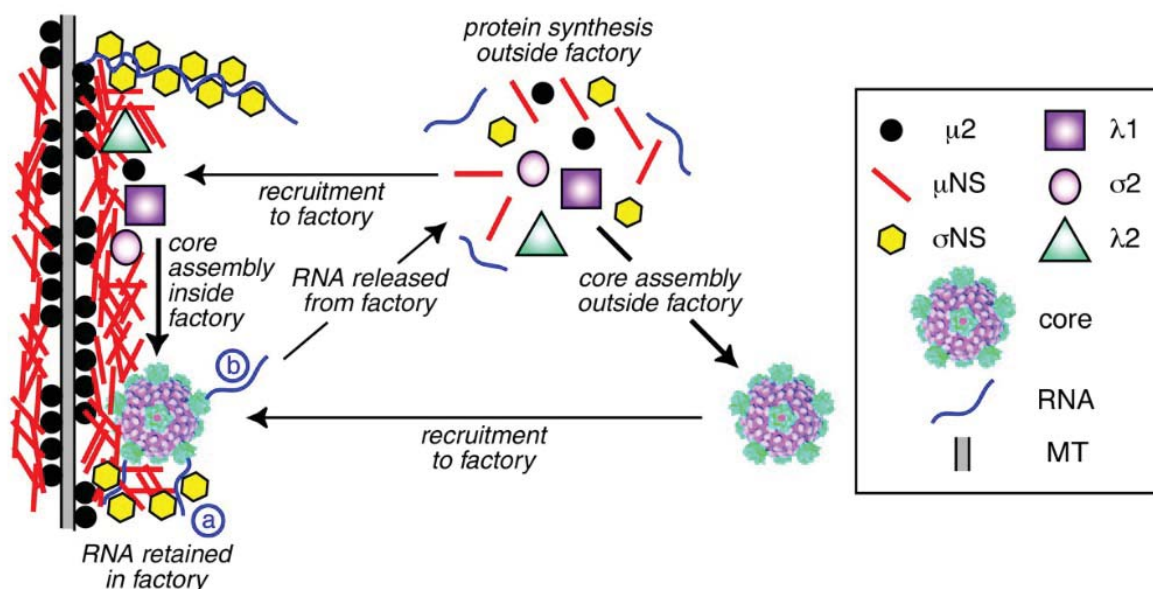


Figure 4. Formation of viral factories.

The model illustrates the organization of viral factories with a filamentous morphology where μ2 associates the factories with microtubules (MT), and μNS functions as the recruiting protein. Ribosomes are excluded from the factories, so protein synthesis must occur in surrounding regions of the cytoplasm. The core surface proteins and the single-stranded RNA-binding protein σNS are recruited to the factory through association with μNS.

Core assembly is proposed to occur within the factory but might also occur in surrounding regions of the cytoplasm as shown in the model. Cores assembled in the cytoplasm may then be recruited to the factory through association with μ NS. New plus-strand RNA transcripts produced by μ NS-associated cores within the factory may be largely retained there, possibly by binding to σ NS (a), which may promote their assembly into progeny particles. Some newly produced viral transcripts, however, must be released into the surrounding cytoplasm (b) to promote ongoing viral protein synthesis. Although the various protein associations are shown as direct interactions, there may be unidentified intermediaries. Modified from Broering et al, J Virol 2004 [120].

The viral factory inclusions of RNA viruses consist of viral dsRNA, viral proteins, partially and fully assembled viral particles, microtubules and thinner filaments suggested to be intermediate structures [121]. Viral dsRNA and ssRNA are sensed by the cellular innate immune system as pathogen associated molecular patterns (PAMPs). PAMPs are molecules associated with groups of pathogens, recognized by cells of the innate immune system and thereby has the ability to trigger antiviral responses. In the viral factories, the viral PAMPs are isolated from inducing the activation of cellular innate immune responses [59].

Early in MRV infection, viral factories form as small punctuate structures throughout the cytoplasm that grow in size and become more perinuclear as infection continues. A similar time course development of viral factory-like structures are seen in PRV infected erythrocytes (Figure 5). These structures are important in recruitment of newly synthesized proteins to allow efficient assembly of progeny virus core particles [122]. MRV μ NS and σ NS are present in the earliest detected, synthesized viral protein-RNA complexes in infected cells, and these proteins also form cytoplasmic inclusion structures similar to viral factories when transfected into cells in the absence of viral infection [118]. In addition to being associated with σ NS, μ NS interacts with each of the five structural proteins that make up the core particle (λ 1, λ 2, λ 3, σ 2 and μ 2) in MRV [123, 124].

Studies on the ARV μ NS homolog have reached similar conclusions regarding the role of μ NS in factory formation [125]. The λ A protein of ARV, homolog to MRV and PRV λ 1, is the first structural protein found within viral factories formed by μ NS, followed by packing of four other viral inner capsid proteins [126]. The study propose that the order of recruitment of viral proteins into viral factories follows a sequential pattern and thus, some proteins appear to be recruited initially to factories by association with μ NS, whereas others are recruited subsequently through interaction with yet unknown factors [126].

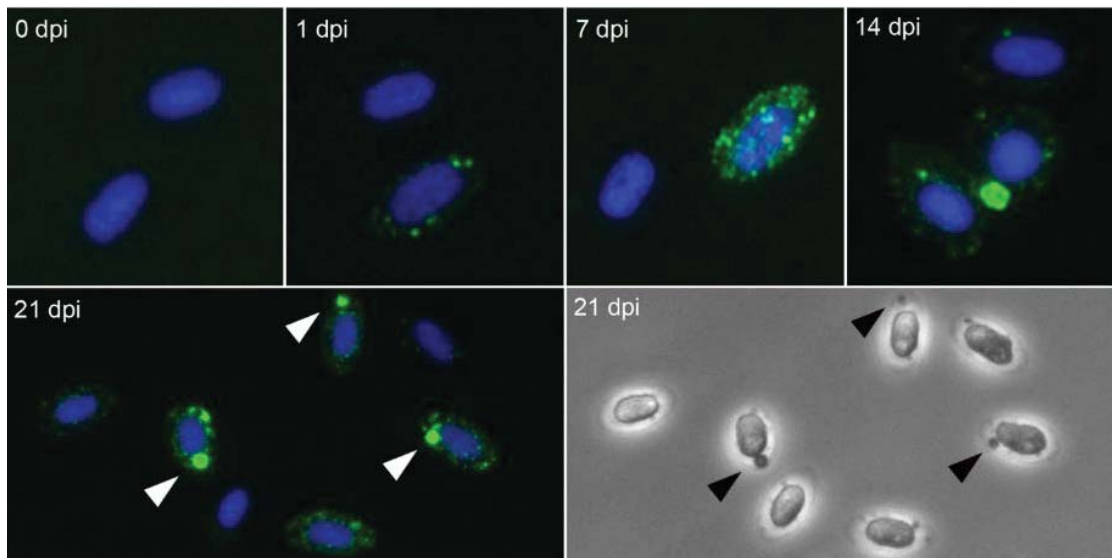


Figure 5. Development of viral factories in PRV infected erythrocytes.

Fluorescent labeling of the PRV σ 1-protein (green) in *ex vivo* infected RBC. The nuclei were stained with Hoechst (blue). Non-infected cells are shown at 0 dpi, and infected cells are shown at 1, 7 14 and 21 dpi. Large PRV inclusions at 21 dpi (marked by arrowheads) was also observed in phase contrast. Modified from Wessel et al, Vet Res 2015 [61].

Two different factory morphologies are described for MRV; filamentous and globular [127]. The difference in morphology are determined by the ability of the virus to interact with the cellular microtubule system and this feature is more specifically mapped to the M1 genome segment encoding μ 2. Only 2 out of 22 MRV strains tested showed the globular factory morphology [127]. Studies propose that the larger surface area of filamentous inclusions allow for more efficient viral replication through better access to small-molecule substrates or newly synthesized proteins from the cytosol [127]. Immunofluorescence and confocal microscopy have been used to identify globular and filamentous inclusions after transfection with non-structural proteins from MRV, ARV and rotavirus. This method has become a valuable tool to probe early steps in the formation of viral inclusions by identifying specific viral proteins [118].

Structures resembling viral factories are also observed in PRV infected erythrocytes (Figure 6) [60, 61]. These resemble the globular structures formed by the MRV T3D strain [60, 127]. The PRV inclusions varies in size from 100 to 1000 nm and are often located in the perinuclear region, encapsulated by a membrane-like structure [60]. Some of the inclusions contain a mixture of reovirus-like particles and a more homogenous material with lamellar structures, while others only contain virus particles [60].

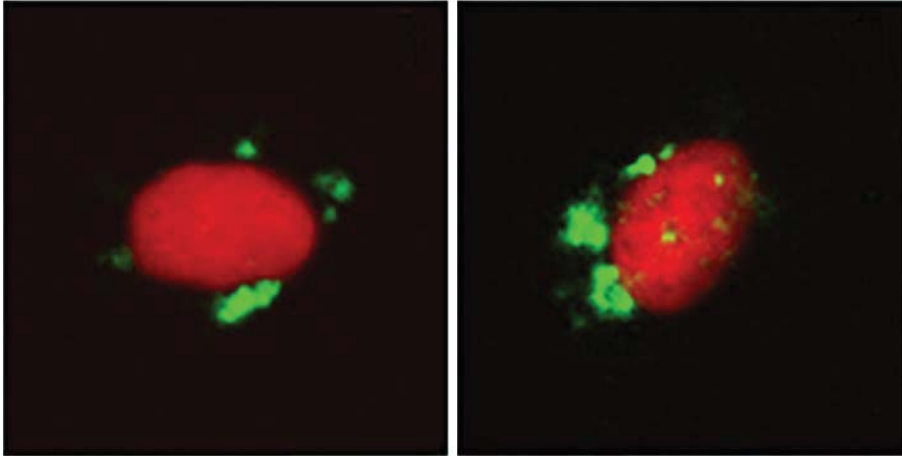


Figure 6. Globular inclusions resembling viral factories.

Green fluorescent labeling of $\sigma 1$ in PRV infected erythrocytes. Nucleus is stained red with propidium iodide. Modified from Finstad et al, Vet Res 2014 [60].

1.3.5. Virion release

The release of progeny MRV particles from infected cells is shown to be associated with cell death and disruption of the plasma membrane [114, 128]. However, viral release from human brain microvascular endothelial cells occurs exclusively from the apical surface via a mechanism that is not associated with lysis or apoptosis of infected cells [129].

It is not known how PRV escape from their target cells. Virus-induced lysis of PRV infected erythrocytes has not been reported following microscopy. Neither has severe anemia been observed in PRV infected Atlantic salmon, indicating that eventual virus-induced lysis of infected erythrocytes is absent, low or at least compensated by erythropoiesis [130]. Interestingly, a variant of PRV was recently demonstrated to be the etiologic agent of EIBS, which is associated with anemia and mass mortality in juvenile Coho salmon [33]. In addition, infection of rainbow trout in fresh water by yet another PRV variant is also associated with anemia and an HSMI-like disease [34].

Extracellular vehicles

During the course of the presented study, an intriguing hypothesis was proposed involving viral utilization of extracellular vehicles for PRV virion release from erythrocytes. Extracellular vehicles are heterogeneous, submicron-sized membrane vesicles capable of intercellular transfer of biological materials and genetic information [131]. Extracellular

vehicles can be found in all bodily fluids and are created by invagination of the endosomal membrane [132-134]. Extracellular vehicles can travel over short or long distances to eventually bind target cell membranes and deliver their cargo [135, 136]. Recent insights shows that viruses exploit extracellular vehicles for several purposes; to enter host cells, to promote viral spread and to avoid humoral immune responses [131]. Apart from incorporating viral proteins, studies have indicated that extracellular vehicles can contain virus-derived nucleic acids, including functional, non-coding microRNAs [137, 138].

The use of extracellular vehicles for virion release would not lyse the PRV infected erythrocytes and could explain the lack of anemia in PRV-infected fish. Several other factors could also indicate that extracellular vehicles might be a plausible mechanism during PRV infection, i.e. the inability of humoral immune response to exterminate infection and difficulties in finding a specific receptor for endocytosis. Preliminary studies on this subject was therefore performed.

1.4. *Orthoreoviruses and effects on host cells*

The mechanisms by which mammalian reoviruses infect susceptible cells in target organs, spreads between the different organ systems and evade host immune response are partly elucidated and knowledge from MRV infection studies is used to understand host responses to PRV. However, how reoviruses ultimately produce disease in the infected host, still remains poorly understood and research in this field is continuously ongoing.

1.4.1. Stress granules

Infection with several MRV strains results in shutoff of host, but not viral, protein synthesis via dsRNA-binding protein kinase R (PKR) activation and subsequent phosphorylation of translation initiation factor eIF2 α [139, 140]. Following inhibition of protein synthesis, cellular mRNAs localize to discrete structures in the cytoplasm called stress granules, where they are held in a translationally inactive state (Figure 7) [139].

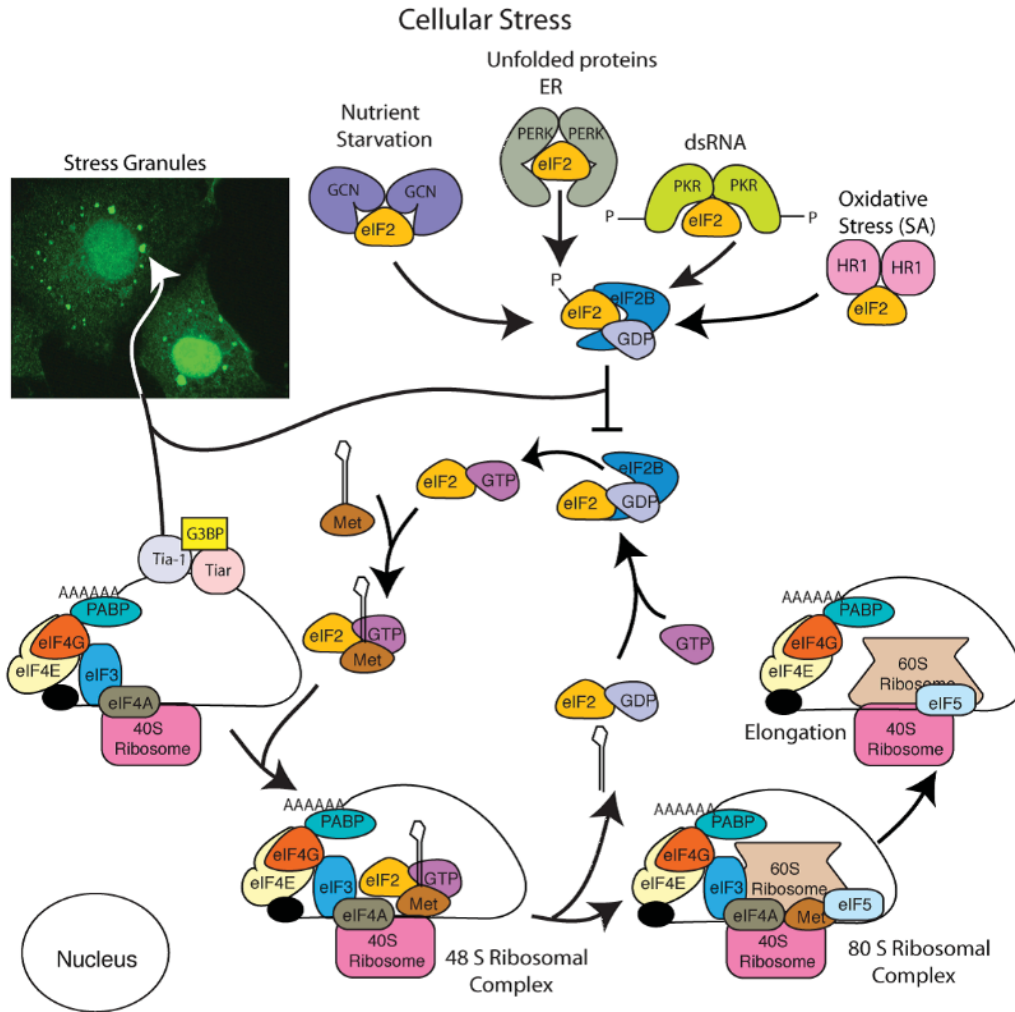


Figure 7. Formation of stress granules.

Model illustrating the formation of stress granules triggered by environmental stress in the cell. Phosphorylated eIF2 α prevents the formation of the ternary complex leading to inhibition of translation of most cellular proteins. Stress granules arise and further inhibit protein translation by sequestering mRNAs and translation initiation factors in a translationally inactive state until the cells recover from stress or undergo apoptosis. Courtesy of Qin Q, Doctoral thesis 2010 [141].

Stress granules are cytoplasmic phase-dense particles consisting of stalled ribosomal complexes where protein synthesis is shut down as a response to environmental stress to conserve anabolic energy for the repair of stress-induced cellular damage [140]. The cellular translation initiation factor eIF2 α is a critical regulatory component of the ternary complex that loads initiator tRNA^{iMet} onto the small ribosomal subunit to initiate protein synthesis. In its phosphorylated condition, eIF2 α is inhibited and translational arrest occurs [142, 143]. Stress granules may also be induced by mechanisms independent of eIF2 α phosphorylation [140].

MRV has the ability to disrupt stress granules despite sustained levels of phosphorylated eIF2 α and interfere with the induction of stress granule formation [144]. The disruption of stress granules correlates with the escape of viral, but not cellular, mRNAs from host translational shutoff [144]. Many aspects of stress granule formation and function are not fully understood, but it is believed that they may serve as part of the innate immune response to viral infections [145, 146].

The structural MRV protein $\sigma 3$ has regulatory functions during replication by affecting the formation of stress granules. [52, 147, 148]. Newly synthesized cytoplasmic $\sigma 3$ binds dsRNA in a sequence independent manner, which inhibits PKR activation and/or the RNase L, and thus prevents translational shutoff [52-54]. Similar dsRNA binding properties are showed for PRV $\sigma 3$, although no specific domain of the protein was found to execute the binding [9, 37].

Some fish species appear to have a PKR-like protein, and in addition a protein with Z-DNA binding domains instead of dsRNA binding domains, called Z-DNA binding protein kinase (PKZ) [149]. It has been demonstrated that recombinant Atlantic salmon PKZ has the ability to phosphorylate eIF2 α *in vitro*, and that wild-type Atlantic salmon PKZ has a direct inhibitory effect on protein synthesis after transient expression in Chinook salmon embryo cells [149]. This implies a role for PKZ, like PKR, in host defense against virus infection [149].

1.4.2. Apoptosis

Reovirus induced apoptosis is a major mechanism of tissue injury in mammals, and involved in the pathogenesis of disease in both the brain and heart [150-152]. In cultured cells, differences in the capacity of MRV strains to induce apoptosis are determined by the S1 and M2 gene segments, and these gene segments are determinants of viral pathogenesis in both the heart and the CNS *in vivo* [153, 154]. Multiple viral gene segments, including M1, L1, L2, and S1, are determinants of MRV-induced acute myocarditis [155, 156].

Apoptotic cardiomyocytes have also been detected in the heart of HSMI diseased fish [157], but is not a common observation [158, 159]. PRV infection typically results in CD8 T cell

myocarditis and red skeletal muscle myositis [158]. Severe anemia has not been reported from HSMI outbreaks in the seawater phase, indicating low, no or compensated virus-induced apoptosis of PRV-infected erythrocytes [130].

1.4.3. Anti-viral response

The immune system is a host defense mechanism comprising many biological structures and processes that detects a wide variety of pathogens. It is customary to divide immune responses into two main categories; the innate immune system and the adaptive immune system. The innate immune responses comprise early immune defense like interferons (IFNs), natural killer (NK) cells and macrophages, while the adaptive immune responses are highly specific for a particular pathogen and are defined by several factors as lymphocytes, antigen receptors and major histocompatibility complex (MHC) type I and II.

Innate immune response

The innate immune response develops as a rapid and regulated defense mechanism triggered by receptors that recognize the conserved patterns of pathogenic organism such as proteins, lipoproteins, dsRNA and other PAMPs, and initiate signaling cascades that in turn directs the innate immune response [160, 161]. The antiviral response is a specific part of the innate immune response triggered by viruses and even very primitive organisms have the ability to mount an innate immune response against pathogens.

In mammals, IFNs can inhibit viral replication by inducing an antiviral state in the host cells or by increasing the antigen-presentation in MHC type I, initiating inflammatory responses, and stimulating the activation of macrophages, NK cells or cytotoxic T cells as well as the differentiation of B cells into antibody-producing plasma cells [160]. IFN is an extremely powerful antiviral response that is capable of controlling several virus infections in the absence of adaptive immunity.

Double-stranded RNA is considered the main PAMP of reoviruses. Fish have two toll-like receptors (TLRs), TLR3 and TLR22, that can bind dsRNA in the endosomes and cytoplasmic membrane respectively [162], and two main cytoplasmic dsRNA receptors; retinoic acid inducible gene (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) [163]. The

interaction leads to activation and nuclear translocation of the transcription factors interferon regulatory factor 3 (IRF-3) and nuclear factor $\kappa\beta$ (NF- $\kappa\beta$), which triggers production of type I interferons, chemokines and cytokines to recruit immune cells to the infection site, and increases expression of MHC type I and co-stimulatory molecules such as CD40, CD80 and CD86 [164]. Type I IFNs are secreted and activate intracellular signaling pathways via a type I IFN receptor, and regulate the expression of a set of IFN-stimulated genes (ISGs). The ISGs include a wide range of genes involved in eliminating viral components from infected cells, inducing apoptosis of infected cells, and conferring resistance to viral infection in uninfected cells [164]. IFNs trigger signal transduction through the JAK-STAT signaling pathway, which results in changes of many cellular pathways including expression of antiviral proteins such as the Myxovirus resistance (Mx) protein, PKR and the virus inhibitory protein Viperin (Figure 8) [165-167].

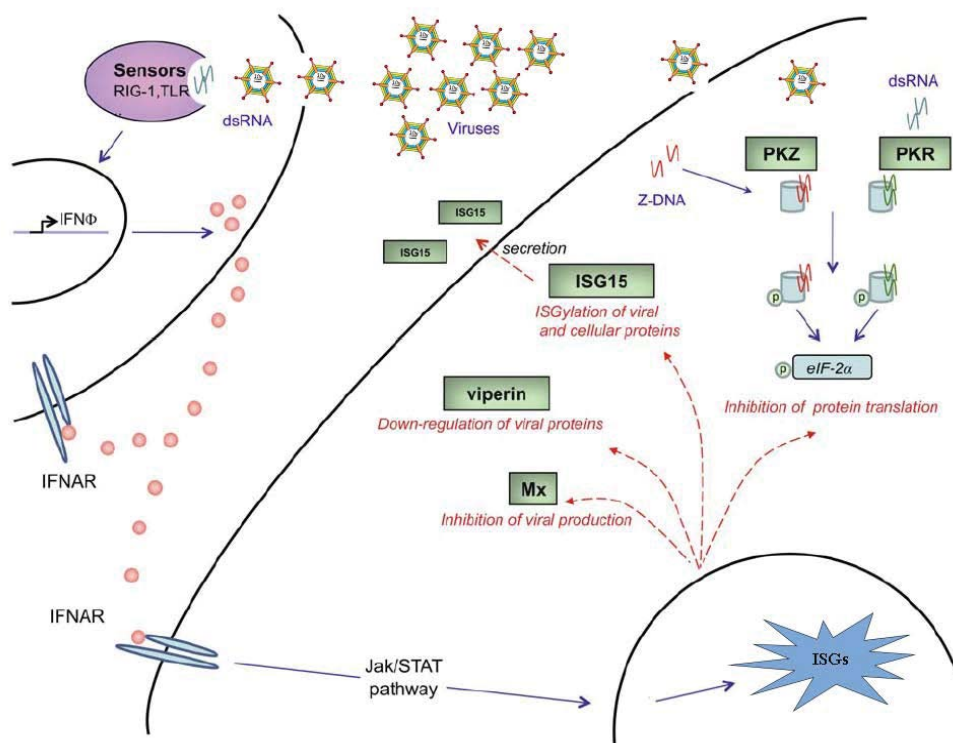


Figure 8. Fish virus induced genes in the context of interferon ϕ signaling.

Following virus entry in the cell, viral nucleic acids bind different sensors as RIG-1 and TLRs and activation of these sensors leads to the induction and secretion of interferons. The cytokines has auto- and paracrine activity mediated through interactions with a transmembrane receptor (IFNAR) that activates the JAK/STAT pathway. This leads to the induction of interferon stimulated genes (ISGs) such as Isg15, PKR, PKZ, Mx or viperin. Modified from Verrier et al, Dev comp immunol 2011 [168].

Viral counter-mechanisms

In order to bypass these antiviral responses, dsRNA viruses have developed counter-mechanisms. During replication of orthoreoviruses, the dsRNA genome is enclosed within the viral core to avoid exposure [169]. In addition, viral RNA can be masked by dsRNA-binding viral proteins, inhibiting the dsRNA signaling pathway, or non-polyadenylated viral mRNA can be produced to omit translational shutdown [144, 170]. Translational shutdown occurs through activation of PKR, leading to phosphorylation of the cellular translation initiation factor eIF2 α and thus blocking cellular translation, while viral RNA translation is maintained [165, 171-173].

Due to structural similarities, PRV may counteract antiviral mechanisms in similar ways as other orthoreoviruses [55, 61]. Recently, it has been shown that PRV σ 3-protein binds dsRNA in a sequence independent manner similar to MRV σ 3 [37]. Nevertheless, MRV induces IFN- β in infected cells [174-176]. This indicates that dsRNA intermediates may be recognized [161].

PRV and anti-viral response

The nucleated piscine erythrocytes have the potential to support viral propagation and contribute to efficient virus distribution in the host organism [69]. The ability to support an innate antiviral response is therefore essential in prevention of disease. PRV infection in erythrocytes has previously been shown to induce expression of type I interferon and interferon-regulated genes, which induces the expression or stimulates a wide range of antiviral effector molecules [61, 159]. In an *ex vivo* study where piscine erythrocytes were infected with PRV, gene expression of IFN- α , Mx, RIG-I and PKR was upregulated, indicating mobilization of an antiviral immune response [61]. A cohabitant study also showed increased expression of several housekeeping and antiviral innate immune genes, such as elongation factor 1 α (EF1 α), β -actin, IFN, IFN-stimulated gene 15 (ISG-15), Mx, PKR, RIG, Viperin and Ubiquitin specific peptidase 18 (USP18), that lasted for more than ten weeks in blood cells, spleen and heart after PRV infection. The level of innate immune gene expression correlated strongly with the Ct of PRV RNA [177]. However, a study performed on PRV from western North America failed to cause an innate antiviral response [178], indicating that there might be strain differences in virulence or differences in host immune response.

Adaptive immune response

The adaptive immune system is a system where the animal acquires long-term protection against a pathogen following encounter. It has been proven that bony fish are able to mount antigen specific immune responses towards viruses similar to what is found in other vertebrates, although important differences exist.

Central to the adaptive immune response are the lymphocytes, divided into B-cells responsible for antibody production, cytotoxic T-cells that directly kill infected or abnormal cells, and helper T-cells that modulate other immune cells through interaction and cytokine production [179]. The adaptive immune response of fish is temperature dependent, and takes longer to develop compared with mammalian counterparts. Also, there are fewer antibody isotypes (IgM, IgD and IgT), with only two being shown to respond to antigen stimulation. Class switching of antibodies is not observed in fish [179]. IgM is the predominant isotype systemically, while IgT predominates in the gut mucosa.

Virus specific antibody responses against several fish viruses, including PRV, have been shown in salmonids [180-183]. A recent report using novel bead-based assays for detection of specific antibodies against PRV proteins, demonstrated a distinct increase in specific IgM against $\mu 1C$ and μNS in plasma of Atlantic salmon during the course of an experimental PRV challenge [183].

T-cells also appear to have an important role in PRV-mediated immune responses. Increased gene expression of the T-cell markers CD8 and CD4, implying mobilization of cytotoxic and memory T-cells respectively, have been demonstrated in PRV-infected salmon hearts [158, 184]. The T-cell mobilization is associated with decreased virus levels in the heart, indicating a protective role. However, the response is also associated with myocarditis [158].

1.5. PRV distribution and disease associations

Several different disease states has been associated with PRV. In Atlantic salmon, PRV causes HSMI and is also associated with the presence of melanised foci in white muscle [7, 55, 185].

In juvenile Coho salmon (*Oncorhynchus kisutch*), a PRV variant called PRV-2, was demonstrated to be the possible etiologic agent of EIBS, a disease that can cause mass mortality [33]. EIBS was first reported in 1982 and screening revealed that the condition was widespread among different Atlantic and Pacific salmonid species, both in freshwater and seawater [186-189]. In rainbow trout, yet another PRV-like virus is associated with anemia, circulatory disturbance and histopathological changes resembling HSMI [34].

1.5.1. PRV in Atlantic salmon

PRV is ubiquitous in farmed Atlantic salmon in Norway a few months after transfer to sea, but can be detected at a lower prevalence in the fresh water pre-smolt stage [78, 190]. The most common method for PRV detection is RT-qPCR analysis [8]. Both the prevalence and viral load in farmed salmon increase following transfer to sea, and then decrease again towards slaughter [78]. This would indicate that the populations goes through a PRV infection where the viral load initially increases towards a peak, before it is reduced again. The virus has also been found in wild returning Atlantic salmon, but it is not known if these fish has acquired the virus through contact with farmed salmon or if it reflects a genuine infection in wild salmon. In addition, PRV has been detected in archival material dating back to the 1970-ies, before the era of Atlantic salmon farming and the occurrence of HSMI [191]. HSMI has so far only been detected in farmed fish [192]. Aquaculture confine animals under high density which generally facilitate transmission of infectious agents and reduce resistance to disease [3].

PRV is geographically widespread, present in wild and farmed salmonid species in Europe, Canada and Chile [78, 192, 193]. The occurrence of HSMI is common in Atlantic salmon in Europe and Chile, the disease however, is not evenly occurring in farmed salmon populations worldwide, and in Canada, only one outbreak of HSMI has been described [194]. An experimental transmission study using PRV-containing material from North America showed that PRV was transmissible to both Atlantic salmon and Sockeye salmon (*Oncorhynchus nerka*), but neither developed HSMI [195]. The reason for this difference is not known, but might depend upon differences in host immune response or viral strains.

1.5.2. Heart and skeletal muscle inflammation

HSMI in farmed Atlantic salmon is caused by PRV and has emerged from its discovery in 1999 to 130-150 annual outbreaks in Norway the last years [4, 5, 8]. HSMI generally occurs 5-9 months after sea transfer and last for several weeks after which the PRV infection persists [196]. HSMI is considered a common disease problem in Norwegian aquaculture with outbreaks all along the coastline [4]. The cumulative mortality varies from negligible to 20%, while the morbidity is almost 100% in affected cages [6]. Infection pressure is an important factor for initiating an HSMI outbreak and stressors such as lice treatment or moving the fish has been observed to precede HSMI [6, 78, 197].

Clinical signs of HSMI include abnormal swimming, anorexia and increased mortality [130]. Typical gross pathologic lesions include signs of circulatory disturbance with pale heart, yellow liver, swollen spleen, visceral petechiae and ascites [130]. The affected fish have severe inflammation and inflammation-associated necrosis in epi-, endo- and myocardium of the cardiac ventricle and atrium, as well as myositis in red skeletal muscle [130]. Diagnosis of HSMI is based on typical histopathological findings in heart and red skeletal muscle (Figure 9) [130, 158]. Histopathological changes have also been observed in other organs [130]. Erythrocytes are often accumulated in the spleen, head-kidney, liver and gills, and both the spleen and head-kidney have higher loads of virus than heart and skeletal muscle during infection [6, 198].

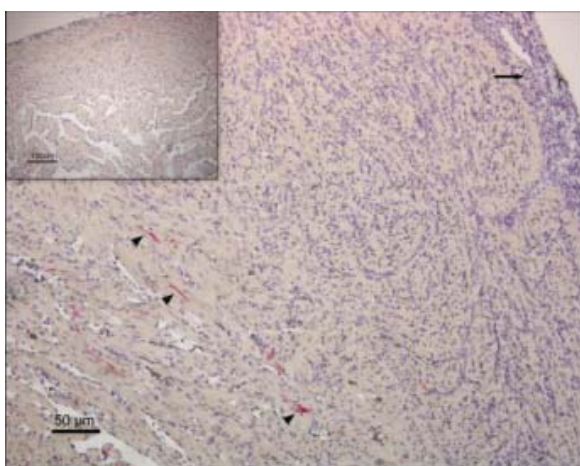


Figure 9. Immunostaining of PRV in heart section from infected salmon.

The illustration shows classical HSMI lesions with epicarditis (arrow) and inflammatory response in the outer part of the compact layer. Immunostaining with μ 1C-antibodies detected PRV outside the area of inflammation

(arrowhead) in the inner part of compactum and in the spongy layer. Courtesy of Finstad et al, Vet Res 2012 [11].

Cardiac lesions in farmed salmon is not uncommon and there are several differential diagnosis to be considered. Both pancreas disease (PD) and cardiomyopathy syndrome (CMS) causes cardiac lesions that have to be differentiated from those seen in HSMI [198-201]. In addition, cardiac lesions can also be related to non-infectious etiology like the combination of restricted activity, continuous food supply, low oxygen levels, crowding, stress and temperature variations [202]. However, many of these assumed relations are stress related and has been described before PRV was known and the fish were deemed non-infected based on the diagnostic machinery available at that time. Due to the ubiquitous presence of PRV in the marine phase, its eventual contribution is difficult to decipher.

1.5.3. Melanised foci in white muscle

Melanised foci in white muscle of farmed salmon has recently been associated with PRV [7]. These are black, brown or red pigments in the muscle fillet and up to 20% of salmon on some farming sites get these spots (Figure 10) [203]. The changes typically appear in the cranioventral and craniodorsal region of the abdominal wall, but may also be found elsewhere in the musculature [204, 205]. The problem exists along the whole coastline and contributes to a large economic loss in the Norwegian aquaculture industry as the fillets with pigmentation are downgraded.

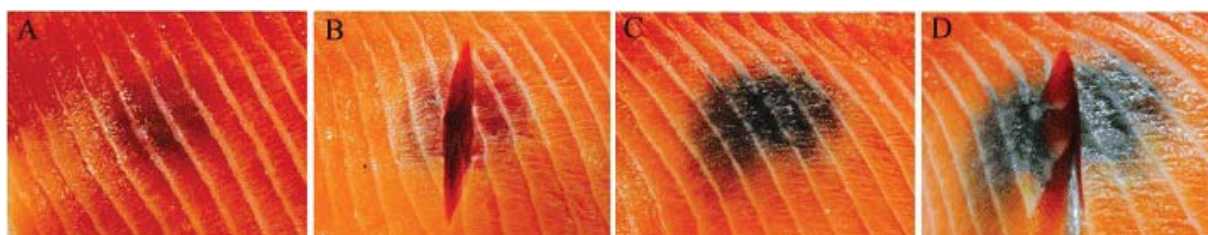


Figure 10. Melanised foci in white musculature of salmon.

The illustration shows melanised foci in white muscle of Atlantic salmon. (A) A red focal change in the muscle of the craniodorsal region of the abdominal wall. (B) An incision through a red focal change showing discoloration extending deep into the fillet. (C) A melanised focal change detected in the same anatomical location in a different fish. (D) A melanised focal change with extensive amounts of a whitish tissue extending into the depth of the fillet. Modified from Bjorgen et al, Vet Res 2015 [7].

The pigment is attributed to melanin-producing leukocytes referred to as melano-macrophages [206, 207]. It has been suggested that the melanin foci occur due to chronic inflammation induced by dislocation of oil-adjuvanted vaccine components, but later studies has showed that also unvaccinated fish may develop similar lesions [204, 208]. Mechanical trauma preceding focal intramuscular hemorrhage has also been suggested as cause, but are not supported by histological investigations.

Histological investigations in a recent study revealed that red focal changes were dominated by hemorrhages and myocyte necrosis, consistent with acute manifestations of muscle damage, while the focal melanised changes were dominated by granulomatous tissue and melano-macrophages, indicating a chronic inflammatory response [7]. In addition, the study showed that large amount of PRV antigens detected by immunohistochemistry was the common denominator to all red and melanised changes, suggesting that PRV has an explicit role in the formation of melanised foci [7].

1.5.4. Fish welfare

In Norwegian salmon farming, approximately 15-20% of the fish transferred to sea die before they finish production cycle, which today equals approximately 50- 60 million fish annually lost during seawater production [209]. Current evidence suggests that fish are capable of perceiving sensory stimuli and experience feelings like fright, pain and discomfort and fish are protected by the Animal Welfare Act along with other vertebrate species, decapods, octopus, squid and honeybees [4, 210, 211].

Diseases affect welfare in various ways depending on which organs and functions that are affected, and the intensity and duration of the pain or discomfort accompanying the disease [4]. HSMI causes severe inflammation in the heart and skeletal muscle, and severe muscle pain is likely to accommodate this condition. Surviving fish seem to recover, but they may have cardiac lesions for month after an outbreak [6]. With the ubiquitous presences of PRV and only sparse knowledge on how the virus causes disease, PRV poses a tremendous risk for reduced welfare for farmed salmon.

Research institutions and fish health personnel have a special responsibility to work for improved fish welfare and influence the attitudes towards fish in both the aquaculture industry and among the general public [4]. As good health is a prerequisite for good welfare, efforts dedicated to improve fish health are important for welfare [4].

1.6. Vaccine against HSMI

Considering the increasing occurrence of viral disease outbreaks in Norway and the discovery of HSMI in countries such as Canada, Scotland and Chile, diseases associated with PRV exhibit a considerable risk for the aquaculture industry [178, 192, 193, 212, 213]. If the estimated growth in aquaculture sustains, proper disease control is required [1].

The load of PRV increases in a population after transfer to sea and the total load of virus at peak of infection may be a key factor in disease development. Currently, there is no treatment to reduce the extent of an HSMI outbreak. Increased survival has been observed feeding tetradecylthioacetic acid (TTA) and functional feed may be an alternative to reduce the extent of an HSMI outbreak [214]. Breeding for increased disease resistance against infectious pancreatic necrosis (IPN) in Atlantic salmon has been successful, and a similar approach will probably be tested against HSMI. A good solution would nevertheless be a vaccine that potentially could bring the total PRV load below threshold for HSMI outbreaks.

Vaccines administered to Atlantic salmon in Norwegian salmon farms aim to give protection in the grower stage in sea-water. Classical inactivated, vaccines are based upon the ability to cultivate the respective pathogen. Commercially available vaccines for Atlantic salmon are administered as oil-adjuvanted intra peritoneal (i.p.) injections containing inactivated virus particles from cell cultures or recombinantly produced capsid proteins [215, 216]. The study of molecular mechanisms linked to PRV infection has been limited by the lack of susceptible cell lines for cultivation of the virus, which hampers both research on the virus and the ability to make a traditional vaccine. However, progression in vaccine development has moved from using whole microorganisms to subunit vaccines that contain only the proteins of the pathogen's that induce protective responses. Subunit vaccines are often less immunogenic

than whole pathogens, but incorporation of antigens into biomaterials can achieve a desired vaccine response.

One of the most promising vaccine preparations against viral diseases is DNA vaccines. Naked plasmid DNA results in gene expression of viral proteins in the muscle tissue of vaccinated fish and several different plasmid backbones are available for use as DNA delivery systems [217]. A Salmonid Alphavirus (SAV) replicon vector, based on a virus adapted to cold water piscine hosts, has received considerable attentions in fish vaccines studies and found to induce efficient protection against infectious salmon anemia (ISA) and pancreas disease (PD) in challenge experiments [216, 218-221]. Several other DNA vaccines based on different plasmid backbones has also been proven efficient in a large number of fish species and against a variety of viral diseases [222-224]. Still, only one DNA vaccines for aquaculture fish are licensed for sale. This DNA vaccine induce protection against infectious hematopoietic necrosis (IHN) virus and has been used in Canada with great success since 2005 [225]. Recently, a DNA vaccine against PD has also been recommended for use in salmon in Europe (Press release, European Medicines Agency).

For MRV, monoclonal antibodies directed against outer capsid proteins $\sigma 1$, $\sigma 3$ and $\mu 1C$, as well as core protein $\lambda 2$, can neutralize the virus [226]. The corresponding proteins in PRV might be suitable vaccine candidates. In addition, intracellular PRV proteins may induce a cellular, T-cell mediated response against PRV-infected cells. A vaccine against PRV may have to induce both these pathways for optimal protection. Controlling HSMI by vaccination will significantly benefit both the fish health and welfare, and the economics in Atlantic salmon farming.

2. Aims of study

Main objective

The principal aim of the present thesis was to contribute to knowledge regarding the function of PRV proteins. The thesis encompasses both *in vitro* studies of protein functions as well as *in vivo* challenge experiments for studies of protein kinetics.

Sub-goals

1. Function of PRV virus factory assembly proteins

Based on comparative data from MRV and TEM analysis of PRV infected piscine erythrocytes, we hypothesized that the μ NS protein is the main organizer in assembling progeny particles. The primary goals in this study was to examine the subcellular localization of the μ NS protein and its interaction with other PRV proteins.

2. PRV protein kinetics in blood cells

*In this study, we investigated the kinetics of PRV in blood cells from experimentally infected fish. We hypothesized that PRV causes an acute massive infection of a relatively short duration. In addition, we further investigated our *in vitro* results from sub-goal 1 and studied μ NS and the formation of viral factories *in vivo*.*

3. DNA vaccine expressing PRV non-structural proteins

In this study, the protective effect of DNA vaccines expressing the non-structural, factory forming, PRV proteins was tested in experimental challenges. We hypothesized that expression of PRV μ NS would induce a protective host immune response against HSML.

3. Summary of papers

Paper I

The non-structural protein μ NS of *Piscine orthoreovirus* forms viral factories

Haatveit HM, Nyman IB, Markussen T, Wessel Ø, Dahle MK, Rimstad E.

Veterinary Research 2016, 47:5

In this study, the subcellular location of PRV μ NS, and its co-localization with other PRV proteins was investigated. The PRV μ NS protein forms dense globular cytoplasmic inclusions in transfected fish cells, resembling viral factories. In co-transfection experiments with μ NS, the σ NS, μ 2 and λ 1 proteins were recruited to the globular structures. Immunoprecipitation and subsequent western blot analysis confirmed the association between μ NS- σ NS and μ NS- μ 2. Expression of the N-terminal 401 amino acids of the μ NS protein did not form viral factory-like structures, mapping this feature to the remaining C-terminal 351 amino acids. The findings strongly suggests that μ NS is the main protein involved in formation of viral factories during PRV infection.

Paper II

Viral protein kinetics of *Piscine orthoreovirus* (PRV) infection in Atlantic salmon blood cells

Haatveit HM, Wessel Ø, Markussen T, Lund M, Thiede B, Nyman IB, Braaen S, Dahle MK, Rimstad E.

Viruses 2017, 9, 49

The aim of this study was to investigate the kinetics of PRV infection in Atlantic salmon blood cells. Using an experimental cohabitation challenge model running for 8 weeks, we found that PRV causes an acute infection of blood cells before settling at a lower level of persistence. Maximal viral protein load was observed 5 weeks post challenge by western blotting, decreased sharply thereafter and was undetectable from 7 weeks post challenge. In contrast, viral RNA levels was consistently high. Globular viral factories representing viral

replication were observed in red blood cells from 4 to 6 weeks post challenge and we demonstrated interactions between μ NS and the structural PRV proteins σ 1, μ 1, λ 1 and λ 3 at these time points using western blotting and LC-MS. We also found different size variants of μ NS and the outer capsid protein μ 1 at specific time points during infection. A proteolytic cleavage fragment of the μ 1 protein was the only viral protein detectable 7-8 weeks post challenge, indicating that this μ 1 fragment may be involved in the mechanisms of persistent infection.

Paper III

DNA vaccine expressing the non-structural proteins of *Piscine orthoreovirus* induces moderate protection against heart and skeletal muscle inflammation in Atlantic salmon (*Salmo salar*)

Haatveit HM, Hodneland K, Braaen S, Hansen EF, Frost P, Rimstad E.

Manuscript

In this study, DNA vaccines expressing the non-structural protein μ NS in combination with other PRV proteins were tested in two experimental cohabitant challenges. We used two different plasmid backbones; one containing the replicon machinery of *Salmonid alphavirus*, the pSAV replicon vector, and one conventional vector, pcDNA3.1, where the expression of proteins is controlled by the cytomegalovirus promoter. *In vitro* expression analysis using CHSE cells showed that the transfection efficiency was substantially higher for the pcDNA3.1 vector than the pSAV replicon vector. Results from the experimental challenges demonstrated that most DNA plasmid combinations did not induce significant protection against HSMI in Atlantic salmon, but modest protection was obtained with the vaccine based on the pcDNA3.1 vector expressing the combination of μ NS, σ NS and σ 1.

4. Results and discussion

PRV was first discovered in 2009 and upon initiation of this study, published information on PRV was limited. During the last years, the virus has been characterized and found to belong to the genus *Orthoreovirus*, subfamily *Spinareovirinae*, family *Reoviridae*. Furthermore, the etiological association with HSMI in Atlantic salmon has been confirmed; salmon erythrocytes was reported to be a major target cell type for PRV, enabling development of an *ex vivo* virus cultivation system using piscine erythrocytes, as well as purification of the virus [5, 9, 11, 60, 61].

However, basic molecular studies on PRV replication in target cells are still sparse and mechanisms of disease development, strain variations and so forth have not been elucidated. This, in addition to the lack of a proper cultivation system, has also delayed the development of a vaccine against PRV infection and HSMI.

The study of PRV proteins, their role in replication as well as the development of a vaccine against PRV became the main scientific focus of this thesis work.

4.1. *The non-structural protein μ NS forms and organizes viral factories*

The initial study investigated the formation of viral factory-like structures that are observed in PRV infected Atlantic salmon erythrocytes (Paper I). The viral protein μ NS is the primary organizer of factory formation for MRV, and bioinformatics studies had earlier indicated that the PRV analogue of μ NS is encoded by PRV gene segment M3 [55].

Viral factories represent a safe haven where replication, packaging and assembly of novel viral particles can occur shielded from the cellular virus-associated-molecular-pattern detection system and subsequent antiviral response [59]. In this study, the subcellular location of PRV μ NS and its co-localization with other PRV proteins was investigated. PRV μ NS forms dense, globular, cytoplasmic inclusions in transfected fish cells, resembling the viral factory-like structures in PRV infected piscine erythrocytes (Figure 11) [60]. This distribution

pattern was different from that previously described for recombinantly expressed $\sigma 1$, $\sigma 3$ and $\mu 1$ [11, 37, 60], and was neither observed when the non-structural protein σNS , or the structural proteins $\lambda 1$ and $\mu 2$ were expressed in transfected EPC cells. All other PRV proteins were evenly distributed in the cytoplasm (Figure 11).

For MRV, viral inclusions with different morphologies have been observed; filamentous inclusions in the MRV T1L strain and globular inclusions in the MRV T3D strain [127]. The morphologic differences are determined by the ability of the virus to interact with the microtubule system, a feature mapped to MRV $\mu 2$ [127]. In the filamentous factories, MRV $\mu 2$ co-localize with the microtubule system when expressed in cells in the absence of other viral proteins [118]. In a preliminary study on EPC cells transfected with PRV $\mu 2$ and stained with both phalloidin and anti-tubulin- α , two different markers for the microtubule system, such co-localization was not observed (own data, not published). This finding is consistent with the findings from MRV T3D.

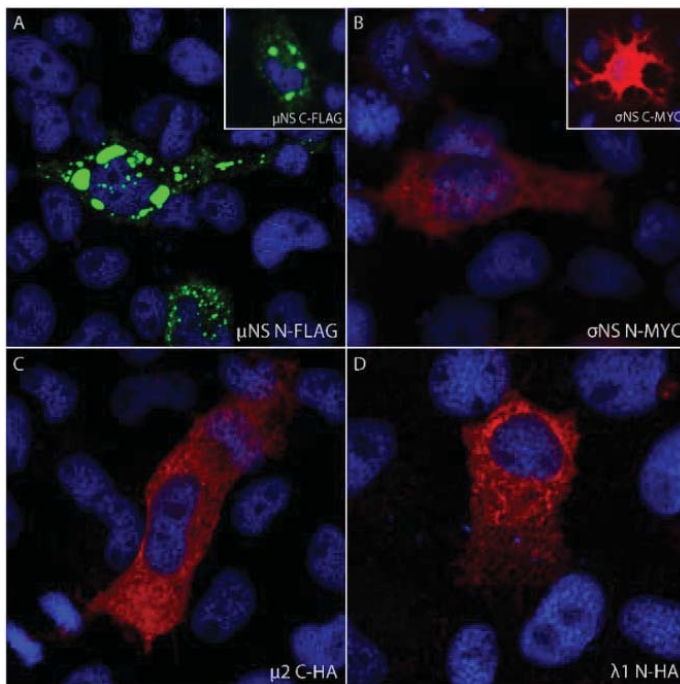


Figure 11. Subcellular localization of PRV proteins.

EPC cells transfected with four different PRV plasmid constructs expressing μNS , σNS , $\lambda 1$, $\mu 2$, respectively, processed for fluorescence microscopy at 48 hpt. (A) EPC cells expressing μNS N-FLAG. Boxed region in top left corner shows EPC cells expressing μNS -C-FLAG. (B) EPC cells expressing σNS N-MYC. Boxed region shows σNS -C-MYC. (C) EPC cells expressing $\mu 2$ -C-HA. (D) EPC cells expressing $\lambda 1$ -N-HA. From Paper I.

MRV μ NS and σ NS are found in the earliest detectable viral protein-RNA complexes in MRV infected cells and form cytoplasmic inclusions similar to viral factories seen during transfection experiments with μ NS [118]. Furthermore, MRV μ NS interacts with each of the five structural proteins that make up the core particle (λ 1, λ 2, λ 3, σ 2 and μ 2) during replication [123, 124]. In co-transfection experiments with PRV μ NS (Paper I), the σ NS, μ 2 and λ 1 proteins were recruited to the globular structures (Figure 12). Immunoprecipitation and subsequent western blot analysis confirmed the association between μ NS- σ NS and μ NS- μ 2. The association between μ NS- λ 1 could not be confirmed, however confocal images clearly proved redistribution of λ 1 when co-expressed with μ NS (Figure 12). The protein-protein interaction between μ NS and λ 1 might be of lower affinity than between μ NS and the other two PRV proteins studied. Viral factories are complex structures and several other factors likely contribute to the protein interactions.

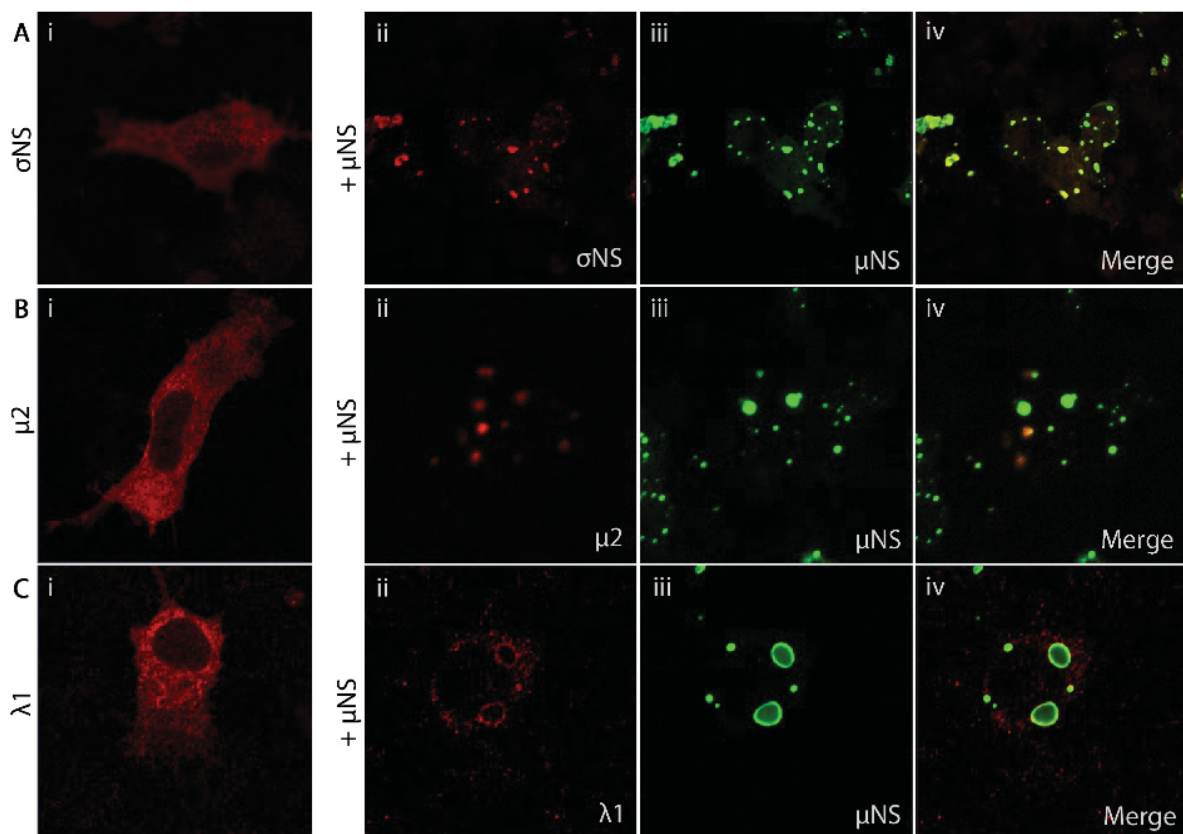


Figure 12. Subcellular localization of PRV proteins after co-transfection with μ NS.

EPC cells transfected with constructs encoding σ NS, μ 2 and λ 1 separately, and co-transfected with μ NS. The cells were processed for confocal microscopy at 48 hpt. (A) EPC cells transfected with σ NS alone (i) and cotransfected with μ NS (ii-iv). (B) EPC cells transfected with μ 2 alone (i) and cotransfected with μ NS (ii-iv). (C) EPC cells transfected with λ 1 alone (i) and cotransfected with μ NS (ii-iv). From Paper I.

The C-terminal of MRV gene segment M3 has four distinct regions comprising 250 amino acids that are sufficient to form viral factories, including two predicted coiled-coil domains, a linker region containing a putative zinc hook between the coiled coils, and a short C-terminal tail region [123]. Deletion of the C-terminal 8 amino acids results in a diffusely distributed protein [125]. PRV μ NS also has a high α -helical content in the C-terminal region, although amino acid sequence homology to MRV is very low, and only 17% identical when comparing the whole μ NS sequence [9]. To further study the PRV μ NS protein and its ability to form globular inclusions, we constructed four truncated variants for expression analysis. In contrast to MRV μ NS, two of the truncated PRV μ NS variants which had deletions of the C-terminal 10 or 17 amino acids, still formed globular, viral factory-like structures when expressed in EPC cells. Deletion of the C-terminal 351 amino acids however, resulted in diffusely distributed protein and no viral factory-like structures, indicating that the feature of viral factory formation is mapped to the C-terminal end. Deletion of the N-terminal 401 amino acids also influenced the formation of globular inclusions, but still irregular structures exhibiting viral factory-like morphology accumulated.

4.2. PRV causes an acute infection in blood cells

In our second study, we set out to investigate the kinetics of viral RNA, viral protein and antiviral immune responses in blood cells from experimentally PRV infected Atlantic salmon (Paper II). In order to perform this time course study optimally, two new antisera were generated from immunization of rabbits with recombinant PRV μ NS and λ 1 proteins, expressed from the ORFs of PRV gene segments M3 and L3 in Sf9 insect cells and *E.coli*, respectively. The raised antisera against μ NS and λ 1, in addition to three other earlier generated antisera against PRV structural proteins [11, 60], were used for analysis of viral protein expression.

Results from RT-qPCR targeting genome segments S1, M2 and M3 revealed a high PRV load in blood during most of the 8 week long experimental cohabitation challenge. Viral RNA was first detected in blood cells 3 wpc and peaked 5 wpc (Figure 13). After the initial peak, M2 and M3 RNA persisted at a high level throughout the study, while S1 RNA decreased. Analysis of viral proteins by flow cytometry showed that the μ NS and the σ 1 proteins were

first detected at 4 wpc, decreased at 5 wpc and were undetectable from 6 wpc and throughout the study.

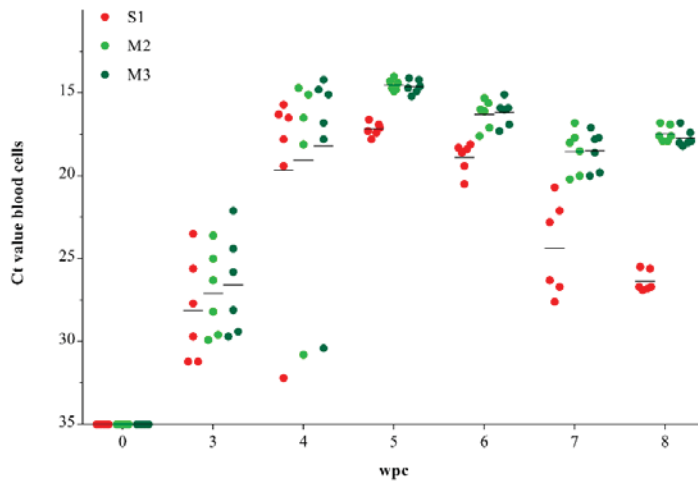


Figure 13. PRV RNA load in blood cells.

RT-qPCR of PRV gene segments S1, M2 and M3 in blood cells from cohabitant fish. Individual (dots) and mean (line) Ct-values, n = 6 per time-point. wpc = weeks post challenge. From Paper II.

The peak in PRV replication was accompanied by induction of antiviral gene expression. The amount of viral RNA correlated with the antiviral response in individual fish before and in the peak phase, but not post peak phase. The continuous high level of M2 and M3 RNA may imply that the antiviral immune response primarily inhibits replication post transcriptionally, in line with the functions of PKR and ISG15 in regulation of translation and protein stability, respectively [227, 228]. Expression of interferon-regulated genes in response to PRV infection in erythrocytes have been reported earlier [61, 159]. The peak phase of virus replication in blood cells occur 2-3 weeks before the expected onset of HSMI. The HSMI lesions are dominated by influx of CD8 positive lymphocytes, indicating that immune mediated responses to the virus infection, and not the cytopathic effect of the virus replication per se, cause the observed myocarditis [158]. Virus-mediated cell lysis is not a common finding in PRV-infected fish, indicating that PRV has other mechanisms of dissemination between target cells and organs.

Immunofluorescent microscopy demonstrated the presence of viral factories in blood cells harvested 4, 5 and 6 wpc. The cells were intracellularly stained with anti- μ NS and anti- σ 1, and the staining pattern for both proteins resembled globular inclusions that varied in size and number. At 6 wpc, the number and size of the inclusions were considerably reduced compared

to peak infection at 5 wpc, and no inclusions were observed after 7 wpc. The cytoplasmic globular viral factories produced by PRV *in vivo* resembled those of the MRV T3D strain and contained the μ NS protein, in line with the observation from recombinant protein expression in paper I.

Corresponding with the timing of viral protein production and observation of viral factories, TEM analysis of the blood cells showed lamellar structures that developed into inclusions containing reovirus-like particles from 4 to 5 wpc, while no virus particles could be observed 7 wpc (Figure 14). The different contents in the inclusions may reflect the progress of the infection of the cell, as lamellar structures were observed at earlier time-points than the inclusions containing reovirus particles.

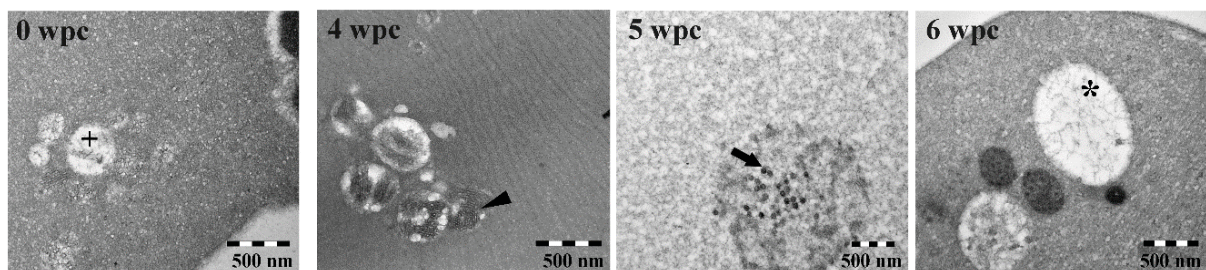


Figure 14. TEM of blood cells.

Electron microscopy of PRV infected red blood cells sampled 0 (negative control), 4, 5 and 6 wpc show small empty vesicles (cross), lamellar structures (arrowhead), reovirus-like particles (arrow) and large empty inclusions (star). From Paper II.

Immunoprecipitation and subsequent western blot analysis in addition to LC-MS analysis confirmed that PRV μ NS interacts with σ 1, σ 2, σ 3, σ NS, μ 1, λ 1, λ 2 and λ 3 proteins *in vivo*. This strongly suggests that μ NS interacts directly or indirectly with all three λ -proteins, the μ 1 protein, and possibly all four σ -proteins, which is in line with the features of other orthoreoviral protein homologues to PRV μ NS and their role in orchestrating the construction of viral factories [21, 122, 126].

Blood from individual fish harvested 3, 4, 5 and 6 wpc were also analyzed by western blot targeting μ NS, and compared to the levels of M3 RNA of the corresponding individual samples (Figure 15). Our results showed that protein and RNA load correlated well in blood harvested at 4 wpc. However, in blood harvested 5 and 6 wpc, there was no consistency between virus protein and RNA load. This indicates that the commonly used assessment of

PRV RNA level by RT-qPCR for quantification of virus in the fish does not accurately reflect the viral load when performed post the acute phase.

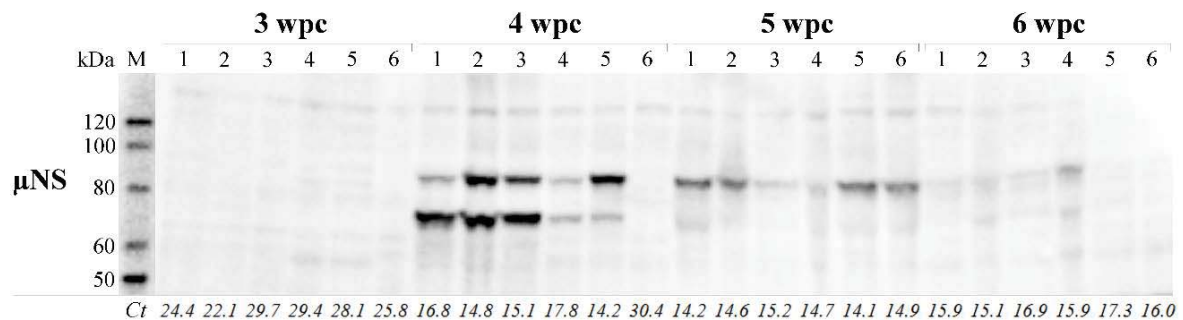


Figure 15. Expression of PRV μNS protein in blood cells.

Blood cells from 3, 4, 5 and 6 wpc (n = 6) analyzed for μNS by western blotting. Ct-values for gene segment M3 (μNS) from the same samples are shown below each lane. M = molecular weight standard; Lane 1 - 6 refers to individual fish (1-6) per time point. wpc = weeks post challenge. From Paper II.

During infections, vast amounts of viral mRNA are produced through the first part of the replication cycle which may be translated in the cytoplasm or confined inside a viral particle (Figure 16). The host innate immune response might interfere with translation leading to a build-up of viral RNA compared to viral proteins and particles. For instance, PKR that is activated by dsRNA and interferon, phosphorylates the eukaryotic translation initiation factor eIF2 α and thus inhibits mRNA translation and viral protein synthesis. The genome of PRV consists of dsRNA, and interferon is induced in PRV infected blood cells [159]. The RT-qPCR used for PRV load estimation in tissue and blood could therefore easily overestimate the viral load, especially in the later stages of infection. In contrast, the load of viral RNA in serum or plasma would probably not be as prone to overestimation, as most RNA detected is particles associated. Western blot and flow cytometry measures viral protein, which are produced during the second part of the replication cycle (Figure 16). This is one step closer to the assembly of novel infectious viral particles, and a better measurement of viral load.

Previous challenge experiments have shown that PRV RNA can be detected by RT-qPCR at a steady level in blood for more than a year after challenge [60, 178], indicating that the fish immune response does not eliminate PRV, and that the infection becomes persistent. In a previous experiment studying the infectious potential of persistently infected Atlantic salmon, sentinel fish were added at 59 weeks post challenge, but no transmission to the sentinel fish

was observed [178]. This indicates that persistently PRV infected fish are not continuously shedding the virus, but maintain a basic production of viral RNA.

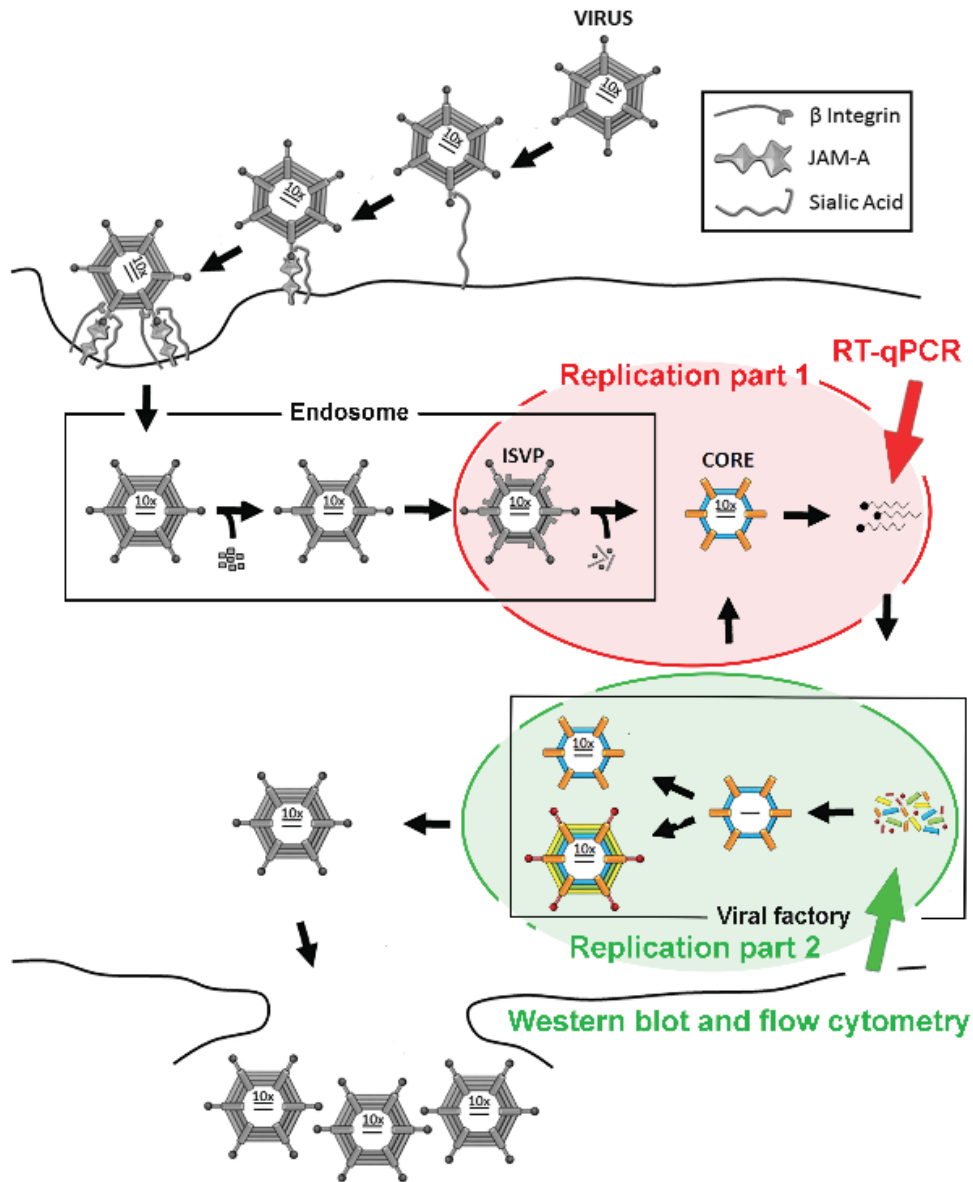


Figure 16. Replication of Mammalian orthoreovirus.

The model illustrates the replication cycle of MRV divided into two separate parts where viral RNA load and viral protein load, respectively can be measured by different laboratory methods. Modified from Mohamed et al, *Viruses* 2015 [44].

Western blot analysis of pooled blood samples from each time-point in the challenge experiment targeting PRV proteins σ_1 , σ_3 , μ_1 , μ_{NS} and λ_1 , showed that all five proteins appeared 4 wpc, peaked at 5 wpc, declined sharply at 6 wpc and were undetectable 7 wpc. The anti- μ_{NS} antibody also detected a band corresponding to a molecular weight of about 70

kDa at 4 wpc, indicating the existence of a smaller size variant of the μ NS protein (Figure 15). MRV μ NS is generated in two size versions by an alternative in-frame translation initiation site in the 5'-end of the gene [229, 230]. PRV M3 has only one ORF corresponding with the Kozak rule of transcriptional activation, although at least two different size variants of the PRV μ NS protein has been predicted considering alternative translational initiation [9]. LC-MS analysis of the smaller size variant of PRV μ NS indicate that a potential internal translation initiation site at M₁₁₅ can generate a μ NSC fragment, homologous to a fragment of the MRV μ NS [56, 229].

Several size variants of the μ 1 protein were detected by western blot at different time-points during the challenge study. At 4 wpc the putative full-length variant (74.2 kDa) was observed. At 5 wpc however, three bands corresponding to approximately 70, 37 and 32 kDa appeared while the putative full-length variant was absent. At 7 and 8 wpc, only one band with the molecular weight of about 35 kDa was detected. MRV μ 1 is cleaved at two positions which generates, in addition to the full-length protein, four different fragments [231]. The N-terminal autolytic cleavage site N₄₂P₄₃, which produces μ 1N and μ 1C fragment, seems to be conserved across orthoreoviruses, including PRV [9, 51, 128]. The band observed 4 wpc on western blot might thus represents the full-length μ 1 protein, while the 70 kDa band detected 5 wpc may represent μ 1C. MRV μ 1 also contains a second cleavage site in its C-terminal region, between Y₅₈₁ and G₅₈₂, which upon cleavage generates the additional fragments δ and ϕ [231]. Although PRV μ 1 only is 28 % identical to MRV μ 1 at the amino acids level [9], the overall predicted secondary structure is similar [45]. LC-MS analysis of the smaller μ 1 variants provided support for a second proteolytic cleavage site at or close to F₃₈₇S₃₈₈, yielding two fragments of 37.7 kDa and 32.1 kDa, close to the sizes estimated from western blot. Thus, our results suggested that the 37.7 kDa and 32.1 kDa bands may represent the PRV homologues of MRV μ 1 fragments δ and ϕ , respectively.

4.3. DNA vaccine expressing the PRV non-structural proteins

The third study aimed at testing the ability of PRV non-structural proteins to induce protective immunity against HSMI after vaccination. The rationale behind this was: 1) There are relatively large amounts of the μ NS protein in infected cells, as seen in the two previous

studies; 2) The μ NS protein is present in inclusion-like structures, and antigens that are not easily degradable may give a long-lasting immune stimulation; 3) PRV has so far proven resistant to cultivation in cell-lines, thus there are no inexhaustible source for correctly folded PRV antigens; 4) Specific antibodies are formed against μ NS in PRV-infected fish, as demonstrated by bead based antibody assays [183], indicating that μ NS could be a suitable PRV antigen.

A DNA vaccination approach was chosen since the non-structural proteins are restricted to the intracellular environment, and protein production in DNA vaccine-transfected cells could mimic this. A DNA vector containing the μ NS ORF was tested alone and in combination with DNA vectors expressing other PRV proteins in a vaccination trial where the protective efficiency against HSMI was studied. The genes for the different PRV proteins were cloned in individual vectors. In addition to testing expression of several different PRV protein combinations, two different expression vectors were tested.

A DNA-layered pSAV replicon vector was compared to a cytomegalovirus (CMV) promoter driven pcDNA3.1 vector as delivery vehicles. Five different vaccine combinations based on the pSAV replicon constructs, all containing the non-structural μ NS protein on a separate plasmid, were developed (Table 4). In addition, four different vaccines based on the pcDNA3.1 expression vector with the conventional CMV promoter were tested (Table 4). The DNA-layered pSAV replicon vector utilizes the machinery of the *Salmonid alphavirus* and induces strong innate immune responses in Atlantic salmon [220]. Antigen-containing pSAV replicon vectors have proven to protect against infectious salmon anemia and pancreas disease in experimental settings [220, 221]. Immunizations with related DNA-layered alphavirus replicons have been shown to robustly activate the cellular and humoral immune responses in mammals [232]. On the other hand, the amount of antigens expressed by the DNA-layered pSAV replicon vectors are lower than conventional CMV promoter driven expression. Initially, there were no intuitive answer to what would induce the best immune response and protection against PRV and HSMI; the stronger innate response induced by the pSAV replicon vector or the larger amount of antigen expressed by pcDNA3.1.

Two separate vaccination trials with Atlantic salmon were performed, both with a 6 week immunization period, followed by PRV infection by cohabitation. The control groups with

mock-vaccinated fish from both trials showed high PRV loads following challenge, indicating successful infection.

Table 4. Overview of the different DNA vaccines prepared for the two experimental challenges.

All vaccines were blinded before shipment to the aquatic research facility. From Paper III.

Trial #1	Vector	Vaccine
1	pSAV	μ NS
2	pSAV	μ NS + σ NS
3	pSAV	μ NS + μ 2 + σ NS + σ 2 + λ 1 + λ 3
4	pSAV	μ NS + μ 1 + σ NS + σ 1 + σ 3 + λ 2
5	pSAV	μ NS + μ 1 + μ 2 + σ NS + σ 1 + σ 2 + σ 3 + λ 1 + λ 2 + λ 3
6	pcDNA3.1	μ NS + σ NS + σ 1
7	pSAV	EGFP (control)

Trial #2	Vector	Vaccine
1	pcDNA3.1	μ NS + σ NS + σ 1
2	pcDNA3.1	μ NS + σ NS + σ 3
3	pcDNA3.1	μ NS + σ NS
4	pcDNA3.1	μ NS
5	pcDNA3.1	EGFP (control)
6	pcDNA3.1	PBS (control)

In vitro expression analysis from the vaccine constructs showed that transfection efficacy was higher, as assessed visually, for the pcDNA3.1 constructs compared to the pSAV replicon constructs. The size of the replicon backbone of 12 073 bp compared to the pcDNA3.1 backbone of 5434 bp could partly explain this, as transfection with larger DNA constructs is less efficient [233].

From the first vaccination trial, RT-qPCR results and histopathology analysis after challenge showed that none of the vaccines based on the pSAV replicon vector induced good protection against HSMI. Still, histopathology lesions consistent with HSMI was slightly reduced in all groups compared to the mock-vaccinated fish. However, the pcDNA3.1 vaccine combination μ NS + σ NS + σ 1 tested in the first challenge experiment altered the kinetics of PRV infection and induced full protection against HSMI. RT-qPCR analysis showed significantly reduced viral loads in blood ($p = 0.002$ at 8 wpc) and a two-week delay in viral RNA peak, while histopathologic examination revealed no lesions in the heart of vaccinated fish (Figure 17A). This clearly indicated the vaccination potential of this triple PRV antigen combination, and the more potent effect of the CMV-driven pcDNA3.1 vector could indicate that the amount

of expressed protein is more important for protection against HSMI than the stronger innate responses induced by alphavirus replicons [234, 235]. PRV is transmitted by cohabitation in the experimental challenges and must cross the mucosal barrier to infect. The differences in efficacy between the pSAV and pcDNA3.1 constructs could be that the induction of protection at mucosal sites or in blocking PRV dissemination to erythrocytes, are related not only to the specific proteins, but also to the level of expression. Consequently, in the second challenge experiment, various constructs using the pcDNA3.1 vector was tested, and the successful pcDNA3.1 vaccine expressing μ NS, σ NS and σ 1 was retested.

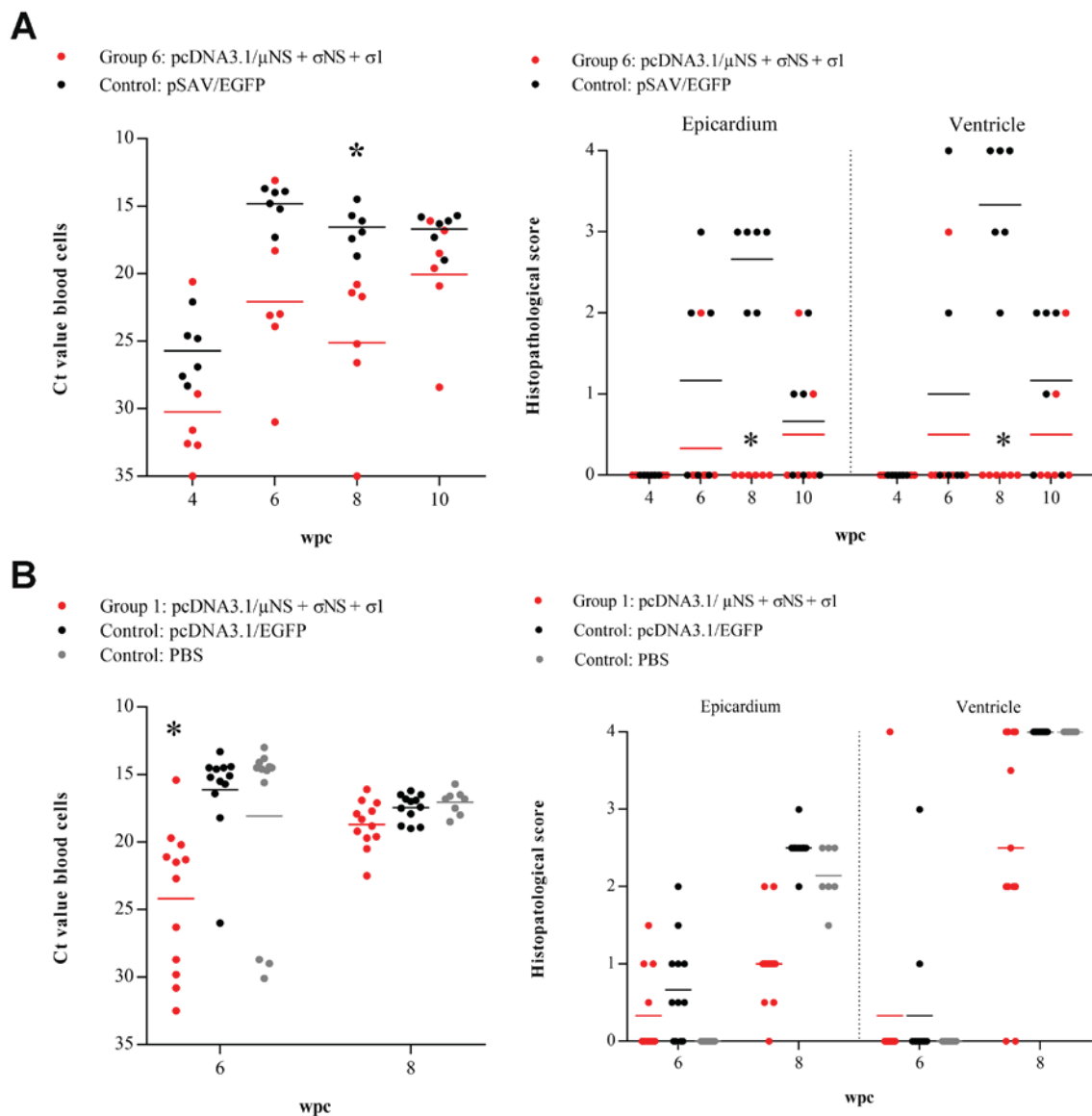


Figure 17. PRV RNA load in blood cells and histopathological score in epicard.

RT-qPCR results (left) and histopathological score (right) comparing fish vaccinated with the pcDNA3.1 vector expressing μ NS + σ NS + σ 1, and fish injected with the mock-vaccine(s) in challenge experiment I (A) and II (B). Individual Ct-values (dots) and mean (line). n = 6/12 per time-point. wpc = weeks post challenge.

PRV RT-qPCR and HSMI histopathology results from the second experimental challenge showed that only the retested vaccine from trial #I induced protection against HSMI. However, the protection was only moderate in this experiment. At 6wpc, the viral RNA load was significantly reduced in the pcDNA3.1/ μ NS + σ NS + σ 1 - vaccinated group compared to the controls ($p = 0.012$ and $p = 0.035$ compared to the pcDNA3.1/EGFP and the PBS groups, respectively). However, at 8 wpc no difference was observed between the groups (Figure 17B). Although histopathological lesions consistent with HSMI were observed after vaccination in the second challenge, the lesions were reduced compared to the control groups (Figure 17B).

The experimental conditions in the two challenges were similar (Table 5), nevertheless, environmental factors cannot be completely controlled. Two different strains of fish were used in the two challenges, but the control fish in both challenges showed similar response to cohabitant infection. Also, although more fish were sampled in the second experimental challenge it included only two time points, which might have reduced the resolution of the viral kinetics.

Table 5. Experimental conditions.

Table illustrating the experimental conditions set for the two separate challenge experiments. wpv = weeks post vaccination; wpc = weeks post challenge. Modified from Paper II.

Fish and management	Vaccination trial #I	Vaccination trial #II
Species	Atlantic salmon (<i>Salmo salar</i>)	
Strain	SalmoBreed Standard	Stofnfiskur
Origin	VESO Vikan Hatchery	
Average weight	25-35 grams	
Physiological status	Presmolts	
Number of fish	290 + 20 % shedders	166 + 20 % shedders
Salinity	Fresh water during immunization/Salt water during challenge	
Stocking density	Max 50 kg/m ³	
Temperature	12°C ± 1°C	
Flow	Adjusted to 70% oxygen saturation	
Water discharge	Tube overflow system	
Cleaning	Once a day	
Photoperiod regime	L:D = 12:12 followed by 24:0	
Feeding	Automatic feeder	
Vaccination	Day 0	
PRV challenge	6 wpv	
Sampling	4, 6, 8 and 10 wpc	6 and 8 wpc
Fish sampled per time-point	6	12

An earlier study has showed that PRV infected fish produce antibodies against both μ NS and μ 1C approximately two weeks post peak viral RNA load, just about the onset of HSMI lesions [183]. The formation of specific antibodies against the non-structural μ NS protein shows the ability of this protein to induce a targeted humoral response. μ NS is a cytoplasmic protein and not a part of the extracellular virus particle, and thus more likely to trigger a cellular than humoral immune response [183]. The PRV μ NS protein forms dense, globular, cytoplasmic inclusions and recruits other PRV proteins for virus replication and assembly (Paper I and II). These cytoplasmic inclusions may trigger a more effective local innate immune response leading to recruitment of immune cells to the vaccination site, with antigen presentation and initiation of long term protection.

All DNA vaccines tested in these two challenge experiments contained the gene for the non-structural μ NS protein. Still, only one of the antigen combinations induced protection against HSMI, indicating that the inclusion bodies formed by μ NS alone do not induce a sufficient immune response to induce protection. Co-expression of the non-structural protein σ NS, which during MRV infection facilitates the assembly of virus particles [118, 120], did not improve protection. However, when combining expression of these to non-structural proteins with expression of the structural virus receptor binding protein σ 1, protection against HSMI was achieved. The mechanism behind this protective immune response has not been elucidated. A potential explanation could be that the non-structural proteins are required to induce the optimal innate immune responses and cytokine/chemokine environment to recruit and activate helper T-cells and B-cells to mount an effective antibody response against σ 1. However, a directed cellular response against μ NS or σ NS-expressing cells may be required as well. Immunity induced by receptor binding virus proteins are classically antibody-based and neutralizing [220, 236].

5. Methodological considerations

During the course of this PhD I have familiarized myself with several different laboratory methods such as molecular cloning, cell culture, transfection, fluorescent microscopy, transmission electron microscopy, production of antibodies, western blot, flow cytometry and RT-qPCR. To generate samples for these studies, I have been acquainted with the use of fish as a research animal and planning of experimental challenges. In addition, I have been introduced to the field of mass spectrometry, although this work was conducted at a different laboratory. The enclosed papers contains detailed information on the use of these methods. The following sections include considerations regarding the material and methods for which I have been mostly involved in during the study.

5.1. *Plasmid construction*

Plasmid DNA is the most common vector for transfecting and expressing novel genes in cell lines. There are several different tools available for construction of plasmids containing the genes of interest, and today gene-containing plasmids can even be synthesized from scratch. In Paper I, we describe the process of constructing plasmids containing the PRV ORFs encoding the μ NS, σ NS, μ 2 and λ 1 proteins.

Topology, size and promotor of the vector construct are all factors that influence the efficiency of transfection and expression in cells, and important to consider before planning plasmid construction. Different promotors can for example be inactive or suppressed in particular cell types, temperatures or conditions. We chose to use a pcDNA3.1 backbone in our plasmid constructs since it contain the conventional CMV promotor that has earlier been proven efficient in transfection of different fish cell lines including those we intended to use (CHSE and EPC). The CMV promotor is also favorable for high transcriptional activity leading to high protein production.

The PRV ORFs of μ NS, σ NS, μ 2 and λ 1 were amplified from cDNA originating from PRV-infected fish. Correct primer design for this amplification step is a key factor for successful cloning. We made sure the primers were specific for the PRV genes and contained a vector

complementary sequence for the upcoming cloning procedure, that no secondary structures or primer dimers were likely to occur, and that the melting temperature (T_m) of both primers was similar. Further, we chose a proofreading polymerase, the PfuUltra II Fusion HS DNA polymerase for this amplification to ensure that the risk for mutations were kept at a minimum. We also considered the length of the product when choosing this enzyme, as the possibility of random mutations during amplification increases with length. After cloning the gene of interest into our selected vector, we transformed bacteria using a traditional heat shock procedure. Electroporation is an alternative transformation method known to be more efficient, however electroporation requires a dedicated electroporator and special cuvettes. Finally, plasmid isolation was performed using a commercial column kit for maxi preps. Purity and quality of the plasmid is critical for a successful transfection as contaminants will easily kill the cells in subsequent transfection studies, and salt will interfere with lipid complexing, decreasing transfection efficiency. Our finalized plasmid constructs were verified by Sanger sequencing.

5.2. *Transfection experiments and epitope tagging*

Flag, myc and HA tags are polypeptides which can be fused to recombinant proteins to enable their targeting by antibodies. Such tags were used to label μ NS, σ NS, μ 2 and λ 1 for the subcellular location study described in Paper I. When initiating this project, we lacked specific antibodies against several of the PRV proteins we wished to study, which led to the use of epitope tags. Epitope-tags helps in identification of proteins in the cell after transfection, as commercial antibodies directed against these tags can be used for immunological detection. Polypeptide tags are usually hydrophilic, which localizes them to the protein surface where they are easily available for antibody binding. However, hydrophilic tags can influence protein function, for example by interfering with a protein-binding site, protein folding or the localization of the proteins in the cell.

In our study, the formation of globular structures by the expressed μ NS protein did not appear to be influenced by cell type, nor C- or N-terminal position of the epitope tag. We performed several control transfections to check for cell viability, determine whether the reporter assay was working properly, and to estimate any insert-related problems. Cell growth conditions

were controlled by including negative controls with no DNA and no transfection reagent, and we used parallel transfections with well described transfection methods as positive controls to establish that the reporter assay was working properly.

We cannot rule out that the inclusion formation induced by μ NS was due to overexpression of misfolded protein, known as aggresomes. The CMV promoter of the pcDNA3.1 vector used is strong and produce high levels of mRNA and protein. However, aggresome formation usually depends on the microtubule system to accumulate perinuclearly [237]. Co-staining of μ NS with both Phalloidin and anti-tubulin- α , designed to stain microtubule components, did not co-localize, indicating that the formation of globular inclusion structures is an intrinsic property of the μ NS protein. This was also confirmed *in vivo* in paper II.

5.3. Production of polyclonal antibodies

The use of epitope tags is a great option for *in vitro* studies of compartmentalization of proteins. However, to confirm our initial results and to further elucidate the kinetics during PRV infection, we needed specific antibodies targeting the different PRV proteins for *in vivo* detection (Paper II). We chose to manufacture polyclonal antibodies (PABs), as opposed to monoclonal antibodies (MAbs), against the non-structural protein μ NS and the structural protein λ 1, during the course of this study.

The technical skills needed to produce PABs is less demanding than for the production of MAbs, and they are inexpensive to produce in comparison. PABs are heterogeneous and produced from a large number of different B cell clones in the immunized host, which allows them to bind a wide range of antigen epitopes on the target protein. They are often more useful than MAbs in techniques such as western blot that include a denaturation step of the target protein, as many MAbs bind to conformational-dependent epitopes. In a standard western blot, the sample and electrophoresis buffer contain the denaturing agent sodium dodecyl sulfate (SDS) and reducing agents like dithiothritol (DTT) that break disulphate bonds within the proteins. This disrupts the secondary and tertiary protein structure in order to allow separation of proteins by their molecular mass in the electrophoresis. However, MAbs generally have a higher specificity and lower background noise, and the use of PABs often

bring along laborious optimization steps. The source of PAb is limited to the serum from the immunized animal, while the MAb theoretically can be produced in inexhaustible quantities from stored hybridomas.

The produced rabbit antisera were based on immunization with recombinant proteins expressed in sf9 insect cells (μ NS) or *E.coli* (λ 1). Both antisera were tested in several different applications, i.e. western blot, flow cytometry, fluorescent microscopy and immunohistochemistry (μ NS, data not published), using recombinant protein in transfected cells and naturally infected cells. One should be aware that neither expression in sf9 insect cells nor *E.coli* can fully mimic the post translational modifications these proteins undergo after expression in PRV infected salmonid cells. The optimal temperature for PRV propagation is considerably lower ($\approx 12^{\circ}\text{C}$) than what is utilized for sf9 insects cells ($\approx 27^{\circ}\text{C}$) or *E.coli* ($\approx 37^{\circ}\text{C}$). The use of recombinant protein might lead to potential loss of protein modifications and conformation-dependent epitopes, which should be taken into consideration when the antibodies are applied in different methods.

5.4. Immunoprecipitation

Immunoprecipitation is the technique of precipitating a protein out of solution using an antibody that specifically targets that particular protein. In both Paper I and Paper II co-immunoprecipitation targeting PRV proteins by the use of antibodies against μ NS from *in vitro* transfected EPC cells or *in vivo* infected blood cells, respectively, was performed. The method is applicable when the proteins involved in the complex bind to each other tightly, making it possible to pull multiple members of the complex out of solution by targeting one member with an antibody. To further investigate the protein-protein interaction, we used western blot to detect the co-precipitated proteins.

There are several considerations that should be regarded when using these methods, including the nature of the antigens and specificity and sensitivity of the available antibodies. The protein-protein interactions might be direct or indirect involving a third protein functioning as a bridge between the targeted antigen and the co-precipitated antigen (Figure 18). The

binding affinities between two proteins might also be below the threshold detectable by the conditions used in the immunoprecipitation- and western blot assays.

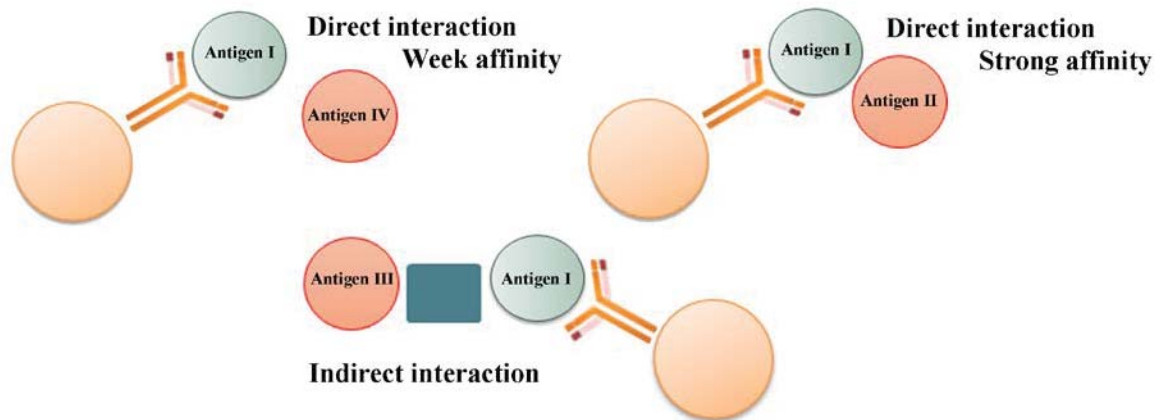


Figure 18. Co-immunoprecipitation.

Model illustrating the complex-formation in co-immunoprecipitation.

5.5. Protein detection

Immunofluorescent microscopy, western blot and flow cytometry are commonly used methods for detection of targeted proteins and depend on specific, selective and reproducible antibodies. Targeting protein in studies of viruses is often more time-demanding than for example detection of the virus genome using RT-qPCR, in addition, protein analyses like flow cytometry requires fresh single cell samples for analysis.

5.5.1. Fluorescent microscopy

In Paper I, cells were fixed, stained and prepared for fluorescent microscopy. Images were captured on an inverted fluorescence microscope (Olympus IX81) and on a confocal laser scanning microscope (Zeiss LSM 710). Fluorescent microscopy is a broadly applicable method generally used to assess both the localization and endogenous expression levels of specifically targeted proteins. There are several considerations to evaluate when using fluorescent microscopy, including the nature of the antigen, specificity and sensitivity of the

primary antibody, properties of the fluorescent label, permeabilization and fixation technique of the sample, and fluorescent imaging of the cells.

Imaging can be performed with fluorophore-conjugated primary antibodies or with non-conjugated primary antibodies targeted by fluorophore-conjugated secondary antibodies. We used a secondary antibody for detection in Paper I and II, which may increase background noise in the images if the secondary antibodies bind unspecific proteins in the cell besides the targeted primary antibodies. However, by adding controls where mock-transfected cells were stained after incubation with either the primary and/or the secondary antibodies, we were able to optimize the protocol and limit background noise.

Another important factor to consider when working with co-transfection and co-staining, is to choose fluorophores that do not overlap in the emission specter. An overlap will make separation between the colors emitted by the two fluorophores difficult, and disturb the interpretation of the result. The secondary antibodies in Paper I was carefully chosen in regard to limit interaction between the fluorescent labels.

5.5.2. Flow cytometry

Flow cytometry is used for analyzing the expression of cell surface and intracellular molecules, characterizing and defining different cell types in a heterogeneous cell population, assessing the purity of isolated subpopulations and analyzing cell size and volume. The method allows simultaneous multi-parameter analysis of single cells, and quantification of the cell fraction containing the specific target proteins.

During this thesis work, flow cytometry was used for detection of viral proteins in blood sampled from Atlantic salmon in Paper II. The non-structural protein μ NS, and the outer capsid protein σ 1 was targeted and flow cytometry measured the fluorescence intensity produced by the fluorescent-labeled secondary antibodies. The blood was prepared in the same manner as for intracellular staining and fluorescent microscopy.

The cell suspension is run through the cytometer and a tiny stream of fluid takes the cells past a laser light one cell at a time. Light scattered from the cells is detected as they go through the laser beam. A detector in front of the light beam measures forward scatter (FS) correlating

with cell size, several detectors to the side measure side scatter (SS) proportional to the granularity of the cells, while fluorescence detectors measure the fluorescence emitted from positively stained cells. In this manner, cell populations can be distinguished based on differences in their size and granularity. In our study, we chose to only detect PRV positive cells in blood and not distinguish between cell types. However, cells were gated according to size and granularity to include only intact cells, and samples from 0 wpc were used as negative controls. Due to slight variation in background staining, the flow charts were gated individually to discriminate between negative and positive peaks. All samples were prepared in duplicates and also stained with corresponding zero serum, anti- μ NS Zero and anti- σ 1 Zero, to check for unspecific binding and reduce background staining.

The sensitivity and specificity of the antibody will affect the outcome of our analysis and several dilutions of the antibodies were tested in the staining procedure to minimize background staining. A weakness in this work was that we fixed, stained and analyzed samples from two of the time-points immediately, while the rest of the samples were fixed and stored for 1 – 2 weeks before analysis. To minimize the potential risk of protein degradation and other storage related problems, we chose not to use flow cytometry as a quantitative measure of protein load, but rather as a qualitative detection system for the presence of targeted PRV proteins.

5.5.3. Western blot

Western blot is used as an analytical technique to detect specific proteins in a sample of tissue homogenate or extract, and provide information on protein mass in kiloDalton (kDa). The method is complex and time consuming and there are several factors to take into consideration. Preparation of the sample, i.e. tissue lysis and denaturation of the proteins, dilutions in regards to sample load and protein detection, and also if you prefer to run the gel under reducing or non-reducing conditions, are only the first step in the protocol in need of several considerations.

The method can be divided into several steps were possible problems awaits; gel electrophoresis, blotting, staining and detection. After sample preparation and gel electrophoresis where you have to choose an optimal gel concentration, running buffer and

running conditions, the blotting of protein to a membrane can start. There are a variety of methods that have been used for this process, i.e. diffusion transfer, capillary transfer, vacuum blotting transfer and electroelution. Electroelution or electrophoretic transfer is the most common method because of its speed and transfer efficiency. Among the electrotransfer systems, you can choose between dry and semi-dry transfer methods, or wet transfer techniques. We chose a wet transfer technique in this thesis work (Paper I, II and III) as this was the equipment available in our lab. Further, having chosen the wet transfer system, you have to consider an optimal membrane type, transfer buffer and blotting conditions. In the staining procedure, the sensitivity and specificity of the antibodies will affect the outcome, and optimization of antibody dilutions can give tremendous effect on the final result. After finalizing the staining procedure, there are also several different available methods for detection. We chose to use chemiluminescence in this work as this method yields the greatest sensitivity.

5.6. RT-qPCR

RT-qPCR is a widely used technique that provides valid information regarding gene expression. However, several factors can influence the quality of data, i.e. variation in sample handling, selection of material, RNA quality and stability, and analytical conditions. The Ct value corresponding to virus detection by targeting the RNA genome or expressed viral gene, is often referred to as a measurement of viral load. However, the quantity of infectious virus particles as detected by end-point dilution in a susceptible cell line would be a more accurate measure of viral load.

RT-qPCR used for detection of dsRNA viruses like PRV in infected cells measure all viral RNA, i.e genomic dsRNA, cRNA and mRNA. In a cell free sample such as serum or plasma, the dominating form of viral RNA is the dsRNA genome in viral particles. An initial denaturation step prior to synthesis of cDNA analysis is crucial for analysis.

Our results from Paper II showed that the level of viral RNA and protein only correlated up until the acute phase of the infection. Post the acute phase, the PRV RNA level, determined by RT-qPCR, did not reflect the level of virus protein in blood. The acute phase of a PRV

infection is relatively short, lasting 4-5 weeks, while the infection lasts at least for more than a year [178], and most probably for a lifetime. Consequently, these findings indicate that measurement of viral load by RT-qPCR has to be interpreted with caution, especially in field samples where the exact time of infection is not known. Furthermore, in several publications we have shown that PRV load peaks 2-3 weeks before HSMI lesions culminate [11, 60]. Combined, these findings indicate that studies of field samples aiming to eluate a relationship between PRV and HSMI based on viral load assessed by RT-qPCR have fundamental methodological weaknesses [78, 238]. This is further complicated by the unknown factor of the relative amounts of mRNA for the different genomic segments of PRV throughout the infection.

5.7. *Experimental challenges*

In Paper III, the conditions for the experimental challenge after vaccination were based on earlier conducted PRV experimental cohabitation challenge studies. We chose a cohabitation route of transmission as this resembles the natural way of infection where the virus must cross the mucosal barrier to infect. On the other hand, mucosal immunity can be difficult to achieve without using replicating vaccines or mucosal vaccine delivery. However, several factors are hard to standardize in cohabitation experiments; the infection pressure in the tank will depend upon the initial infective dose given to the shedders, the immune response of the shedders and the cohabitants; fish density; fish size and age; water flow; and other environmental factors, i.e. in a broad sense the interaction between virus, host and environment (Figure 19).

The number of fish sampled, the number of sampling points, what kind of samples, and how we decide to analyze our samples, will make an impact on the results. We increased the number of sampled fish from Trial I to Trial II in paper III for more accurate statistics. We also chose to reduce the number of sampling points, i.e. reduce the costs. However, the reduction in sampling points was accompanied by an additional sampling of the control fish at 4 wpc, to estimate the peak viral load and thus plan the two sampling points accordingly. We chose to only sample heparinized blood, plasma and heart (stored in 4% formalin and RNAlater), as earlier studies have shown that erythrocytes are important target cells for PRV and the diagnosis of HSMI is based upon histopathologic lesions in the heart.

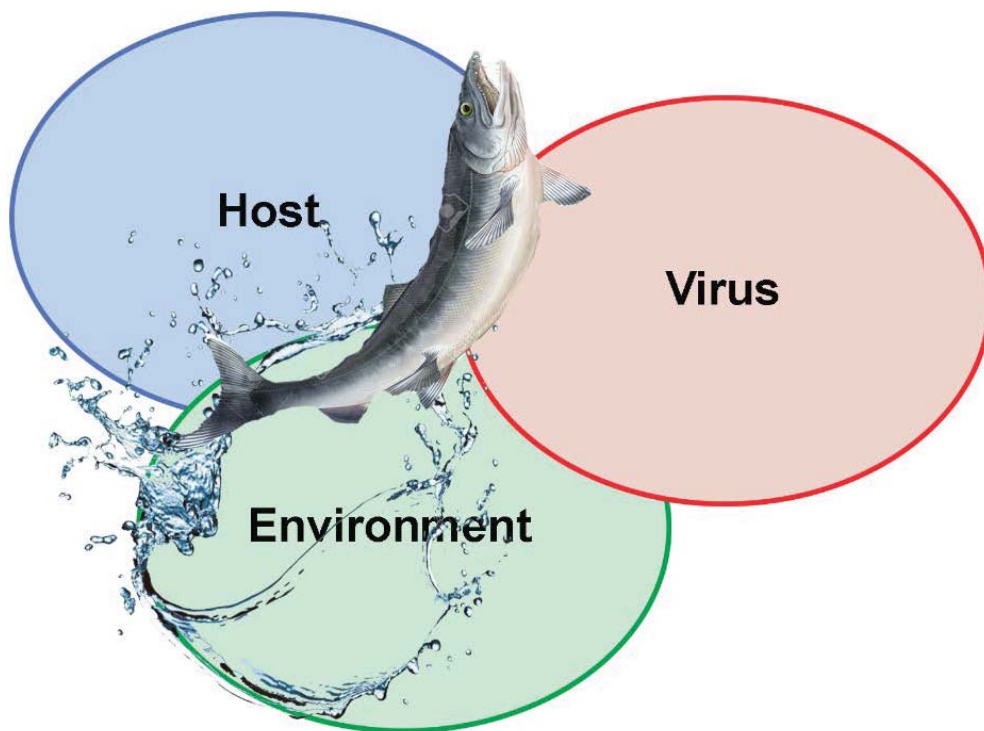


Figure 19. Factors influencing the experimental conditions.

The model illustrates interaction between the main factors influencing the experimental conditions.

In addition, since the aim of this study was to induce protection against HSMI by DNA vaccination with combinations of different PRV proteins expressed from expression plasmids or replicons, we chose to assess histopathology lesions in heart as the diagnostic measurement of HSMI. The histopathologic scores are subjective, and different scales for the scoring of heart lesions have been used in the literature [11, 239]. Analysis from both challenges conducted in this study was performed blinded by the same person using a scoring scheme ranging from 0 to 4.

6. Main conclusions

The results presented in this work have brought us closer in understanding PRV infection and development of HSML.

PRV infection of a cell leads to formation of globular viral factories in the cytoplasm. This formation is organized by the non-structural protein μ NS and is pivotal for early steps in viral replication. The feature of viral factory formation is mapped to the C-terminal 351 amino acids of the μ NS protein. In co-transfection experiments with PRV μ NS, the σ NS, μ 2 and λ 1 proteins were recruited to the globular structures, and as for other orthoreoviruses the majority of virus-encoded proteins localize completely or partially within the viral factories.

PRV causes an acute infection in blood cells. The production of PRV proteins and virus particles in these cells culminates after a relatively short period, i.e. 1- 2 weeks, before the infection moves into a persistent state. Our results show that PRV protein and RNA levels only correlate over a short time-period prior to and during the peak phase of viral replication. Viral factories were observed from 4 to 6 wpc and TEM analysis of infected piscine erythrocytes showed inclusions containing reovirus particles 5 wpc. Immunoprecipitation and subsequent western blot analysis in addition to LC-MS analysis confirmed that the factory generating μ NS protein interacts with σ 1, (σ 2), σ 3, σ NS, μ 1, λ 1, λ 2 and λ 3 proteins *in vivo*. The μ NS protein appears early in infection and exist in two isoforms. The smaller size variant of PRV μ NS is possibly encoded from an internal translation initiation site, generating a μ NSC fragment. Several size variants of the μ 1 protein, probably generated by autolytic and proteolytic cleavages, were also identified and observed at different time points during infection. One cleavage fragment of μ 1 was the only viral protein detectable 7 weeks post challenge (wpc) and onwards, indicating that this μ 1 fragment may be involved in the mechanisms linked to persistent infection.

The μ NS protein is only present in cells during virus replication and has the ability to recruit other PRV proteins and form globular cytoplasmic inclusions. This ability makes μ NS an intriguing protein and our results from a vaccination trial in Atlantic salmon showed that a DNA vaccine expressing the non-structural protein μ NS in combination with the non-structural protein σ NS and the cell attachment protein σ 1, induce moderate protection against HSML.

7. Future perspectives

PRV is a ubiquitous virus well-adapted to marine farming of Atlantic salmon. PRV has been detected in both farmed and wild salmon, but no lesions consistent with HSMI have been found in the wild population [13]. In the last couple of years, different variants or subtypes of PRV, i.e. PRV1, PRV2 and PRV3, have been recognized in the aquaculture industry of salmonids. The virus has been linked to several different disease conditions, i.e. HSMI and melanised foci in white musculature of Atlantic salmon (PRV or PRV1), EIBS in Coho salmon (PRV2), and an HSMI-like disease in rainbow trout (PRV3 or PRV-Om), respectively [7, 8, 33, 34]. Recently, a novel orthoreovirus was associated with epidemic mortality in wild largemouth bass [35]. This implies that there might be variants of piscine orthoreoviruses adapted to several other fish species as well.

The different PRV strains might exhibit differences in virulence, which could explain the lack of HSMI among some populations of Atlantic salmon. Recently, variants of MRV has been found to trigger inflammatory responses that may lead to the development of celiac disease [12], indicating that assumed non-pathogenic orthoreoviruses can have long-term effects not observed during acute infection. The PRV infection might therefore be linked to several, not yet recognized disease conditions. As the virus has a RNA genome, mutations are frequent and due to its segmented nature, reassortments of whole genomic segments are likely to occur. In combination with being ubiquitous in farmed salmon, a small change in virulence can have a tremendous effect on disease outcome. Grass carp reovirus is a serious disease causing virus in the very important aquaculture species grass carp, and although it has 11 gene segments and thus is not an orthoreovirus per se, it is an example of a relatively close relative to PRV with the ability to induce acute disease and high mortality in aquaculture. A virus such as PRV, which is well adapted to farming conditions, and has a segmented RNA genome, should be surveilled for eventual changes in geographical and species distribution, as well as for variations in its virulence.

Erythrocytes are the main reservoir for PRV and the most abundant cell in both the blood and the fish as such. However, erythrocytes are poorly investigated target cells for virus infections, as mammalian erythrocytes are enucleated. This is a basic difference between mammals and cold-blooded animals, and PRV is an example of an infection operating in this schismatic area. Basic molecular studies on PRV infection in erythrocytes might therefore lead to more

information regarding piscine erythrocytes and their functions besides the essential role in O₂ and CO₂ exchange and transport. Recent studies have indicated that erythrocytes might play a role in the fish immune system [67].

More knowledge regarding PRV proteins and their different functions in the virus replication cycle is of central importance for the understanding of this virus, the diseases it may be involved in, and ultimately, for the development of efficient vaccine strategies. The course of this PhD has led to several interesting hypothesis that would be valuable for future research, i.e. the formation of stress granules or the use of extracellular vehicles as an escape mechanism. How is the virus transported from cell to cell after the immune response has gone to action; i.e. during the persistent phase? Can it be shed after the acute phase, and can stressors such as lice treatment induce this? What is the effect of transmission from the aquaculture to the wild salmon population of a low virulent, but ubiquitous virus such as PRV? Can it be utilised as an indicator of the impact of aquaculture on wild fish fauna? Questions that can be raised are numerous and this paragraph is not intended to be exhaustive.

Viral RNA load versus viral protein load is a field that should be further investigated. This should be expanded to other viruses as well. RT-qPCR is the standard method for detecting virus in the aquaculture industry, and as the PRV RNA load does not represent protein load and thereby virus load after peak replication phase, this method should be questioned. Development of more cost-efficient methods for detecting protein load might be a future goal.

In this doctoral thesis, I have studied the acute phase of a PRV infection. Further studies should include the persistent phase as several yet unknown disease conditions might be associated with PRV. For a ubiquitous virus, with unknown consequences of infection, management to control transmission and infection should be prioritized in the future. Vaccines inducing full or partial protection should be further developed and might offer a mitigation.

8. References

1. Moffitt CM, Cajas-Cano L: **The State of World Fisheries and Aquaculture 2014**. *FAO* 2014, **39**(11):552-553.
2. Harvey AC, Juleff G, Carvalho GR, Taylor MI, Solberg MF, Creer S, Dyrhovden L, Matre IH, Glover KA: **Does density influence relative growth performance of farm, wild and F1 hybrid Atlantic salmon in semi-natural and hatchery common garden conditions?** *R Soc Open Sci* 2016, **3**(7):160152.
3. Rimstad E: **Examples of emerging virus diseases in salmonid aquaculture**. *Aquat Res* 2011, **42**:86-89.
4. Norwegian veterinary institute: **Fish health report 2016**. Edited by Hjeltnes B, Walde CS, Jensen BB, Haukaas A. Oslo 2017.
5. Wessel O, Braaen S, Alarcon M, Haatveit H, Roos N, Markussen T, Tengs T, Dahle MK, Rimstad E: **Infection with purified Piscine orthoreovirus demonstrates a causal relationship with heart and skeletal muscle inflammation in Atlantic salmon**. *PloS One* 2017, **12**(8):e0183781.
6. Kongtorp RT, Halse M, Taksdal T, Falk K: **Longitudinal study of a natural outbreak of heart and skeletal muscle inflammation in Atlantic salmon, *Salmo salar* L.** *J Fish Dis* 2006, **29**(4):233-244.
7. Bjorgen H, Wessel O, Fjellidal PG, Hansen T, Sveier H, Saebo HR, Enger KB, Monsen E, Kvellestad A, Rimstad E *et al*: **Piscine orthoreovirus (PRV) in red and melanised foci in white muscle of Atlantic salmon (*Salmo salar*)**. *Vet Res* 2015, **46**:89.
8. Palacios G, Lovoll M, Tengs T, Hornig M, Hutchison S, Hui J, Kongtorp RT, Savji N, Bussetti AV, Solovyov A *et al*: **Heart and skeletal muscle inflammation of farmed salmon is associated with infection with a novel reovirus**. *PloS One* 2010, **5**(7):e11487.
9. Markussen T, Dahle MK, Tengs T, Lovoll M, Finstad OW, Wiik-Nielsen CR, Grove S, Lauksund S, Robertsen B, Rimstad E: **Sequence analysis of the genome of piscine orthoreovirus (PRV) associated with heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (*Salmo salar*)**. *PloS One* 2013, **8**(7):e70075.
10. Day JM: **The diversity of the orthoreoviruses: molecular taxonomy and phylogenetic divides**. *Infect Genet Evol* 2009, **9**(4):390-400.
11. Finstad OW, Falk K, Lovoll M, Evensen O, Rimstad E: **Immunohistochemical detection of piscine reovirus (PRV) in hearts of Atlantic salmon coincide with the course of heart and skeletal muscle inflammation (HSMI)**. *Vet Res* 2012, **43**:27.
12. Bouziat R, Hinterleitner R, Brown JJ, Stencel-Baerenwald JE, Ikizler M, Mayassi T, Meisel M, Kim SM, Discepolo V, Pruijssers AJ *et al*: **Reovirus infection triggers inflammatory responses to dietary antigens and development of celiac disease**. *Science* 2017, **356**(6333):44-50.
13. Garseth AH, Fritsvold C, Opheim M, Skjerve E, Biering E: **Piscine reovirus (PRV) in wild Atlantic salmon, *Salmo salar* L., and sea-trout, *Salmo trutta* L., in Norway**. *J Fish Dis* 2013, **36**(5):483-493.
14. Attoui H, Mertens PP, Becnel J, Belaganahalli S, Bergoin M, Brussaard CP, Chappell JD, Ciarlet M, Del Vas M, Dermody TS: **Family - Reoviridae**. In: *Virus Taxonomy: Ninth report of the international committee on taxonomy of viruses*.

- Edited by King AM, Adams MJ, Carstens EB, Lefkowitz EJ. San Diego: Elsevier; 2012: 541-637.
15. Fields BN, Knipe DM, Howley PM: **Fields virology**, 3rd edn. Philadelphia: Lippincott-Raven Publishers; 1996.
 16. Thalmann CM, Cummins DM, Yu M, Lunt R, Pritchard LI, Hansson E, Crameri S, Hyatt A, Wang LF: **Broome virus, a new fusogenic Orthoreovirus species isolated from an Australian fruit bat**. *Virology* 2010, **402**(1):26-40.
 17. Duncan R: **Extensive sequence divergence and phylogenetic relationships between the fusogenic and nonfusogenic orthoreoviruses: a species proposal**. *Virology* 1999, **260**(2):316-328.
 18. Shmulevitz M, Duncan R: **A new class of fusion-associated small transmembrane (FAST) proteins encoded by the non-enveloped fusogenic reoviruses**. *EMBO J* 2000, **19**(5):902-912.
 19. Key T, Read J, Nibert ML, Duncan R: **Piscine reovirus encodes a cytotoxic, non-fusogenic, integral membrane protein and previously unrecognized virion outer-capsid proteins**. *J Genl Virol* 2013, **94**(5):1039-1050.
 20. Coombs KM: **Reovirus structure and morphogenesis**. *Curr Top Microbiol Immunol* 2006, **309**:117-167.
 21. Schiff LA, Nibert ML, Tyler KL: **Orthoreoviruses and their replication**. In: *Fields virology. Volume 2*, 5 edn. Edited by Knipe DM, Howley PM, Fields BN. Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins; 2007: 1853-1915.
 22. Fukuhara H, Ino Y, Todo T: **Oncolytic virus therapy: A new era of cancer treatment at dawn**. *Cancer Sci* 2016, **107**(10):1373-1379.
 23. Turnbull S, West EJ, Scott KJ, Appleton E, Melcher A, Ralph C: **Evidence for Oncolytic Virotherapy: Where Have We Got to and Where Are We Going?** *Viruses* 2015, **7**(12):6291-6312.
 24. Weiner HL, Drayna D, Averill DR, Jr., Fields BN: **Molecular basis of reovirus virulence: role of the S1 gene**. *Proc Natl Acad Sci USA* 1977, **74**(12):5744-5748.
 25. Sherry B, Schoen FJ, Wenske E, Fields BN: **Derivation and characterization of an efficiently myocarditic reovirus variant**. *J virol* 1989, **63**(11):4840-4849.
 26. Jones RC: **Avian reovirus infections**. *Rev Sci Tech* 2000, **19**(2):614-625.
 27. Rosenberger JK, Sterner FJ, Botts S, Lee KP, Margolin A: **In vitro and in vivo characterization of avian reoviruses. I. Pathogenicity and antigenic relatedness of several avian reovirus isolates**. *Avian Dis* 1989, **33**(3):535-544.
 28. van der Heide L: **The history of avian reovirus**. *Avian Dis* 2000, **44**(3):638-641.
 29. Leland MM, Hubbard GB, Sentmore HT, Soike KF, Hilliard JK: **Outbreak of Orthoreovirus-induced meningoencephalomyelitis in baboons**. *Comp Med* 2000, **50**(2):199-205.
 30. Duncan R, Murphy FA, Mirkovic RR: **Characterization of a novel syncytium-inducing baboon reovirus**. *Virology* 1995, **212**(2):752-756.
 31. Yamanaka A, Iwakiri A, Yoshikawa T, Sakai K, Singh H, Himeji D, Kikuchi I, Ueda A, Yamamoto S, Miura M *et al*: **Imported case of acute respiratory tract infection associated with a member of species nelson bay orthoreovirus**. *PLoS One* 2014, **9**(3):e92777.
 32. Duncan R, Corcoran J, Shou J, Stoltz D: **Reptilian reovirus: a new fusogenic orthoreovirus species**. *Virology* 2004, **319**(1):131-140.
 33. Takano T, Nawata A, Sakai T, Matsuyama T, Ito T, Kurita J, Terashima S, Yasuike M, Nakamura Y, Fujiwara A *et al*: **Full-Genome sequencing and confirmation of the causative agent of Erythrocytic inclusion body syndrome in Coho Salmon identifies a new type of Piscine Orthoreovirus**. *PLoS One* 2016, **11**(10):e0165424.

34. Olsen AB, Hjortaa M, Tengs T, Hellberg H, Johansen R: **First description of a new disease in Rainbow Trout (*Oncorhynchus mykiss* (Walbaum)) similar to Heart and skeletal muscle inflammation (HSMI) and detection of a gene sequence related to Piscine Orthoreovirus (PRV).** *PloS One* 2015, **10**(7):e0131638.
35. Sibley SD, Finley MA, Baker BB, Puzach C, Armien AG, Giehlbrock D, Goldberg TL: **Novel reovirus associated with epidemic mortality in wild Largemouth Bass (*Micropterus salmoides*).** *J Gen Virol* 2016, **97**:2482-2487.
36. Attoui H, Fang Q, Mohd Jaafar F, Cantaloube JF, Biagini P, de Micco P, de Lamballerie X: **Common evolutionary origin of aquareoviruses and orthoreoviruses revealed by genome characterization of Golden shiner reovirus, Grass carp reovirus, Striped bass reovirus and golden ide reovirus (genus *Aquareovirus*, family *Reoviridae*).** *J Gen Virol* 2002, **83**(Pt 8):1941-1951.
37. Wessel O, Nyman IB, Markussen T, Dahle MK, Rimstad E: **Piscine orthoreovirus (PRV) o3 protein binds dsRNA.** *Virus res* 2015, **198**:22-29.
38. Reinisch KM, Nibert ML, Harrison SC: **Structure of the reovirus core at 3.6 Å resolution.** *Nature* 2000, **404**(6781):960-967.
39. Lee PW, Hayes EC, Joklik WK: **Protein sigma 1 is the reovirus cell attachment protein.** *Virology* 1981, **108**(1):156-163.
40. Barton ES, Forrest JC, Connolly JL, Chappell JD, Liu Y, Schnell FJ, Nusrat A, Parkos CA, Dermody TS: **Junction adhesion molecule is a receptor for reovirus.** *Cell* 2001, **104**(3):441-451.
41. Kirchner E, Guglielmi KM, Strauss HM, Dermody TS, Stehle T: **Structure of reovirus sigma1 in complex with its receptor junctional adhesion molecule-A.** *PLoS pathog* 2008, **4**(12):e1000235.
42. Reiter DM, Frierson JM, Halvorson EE, Kobayashi T, Dermody TS, Stehle T: **Crystal structure of reovirus attachment protein sigma1 in complex with sialylated oligosaccharides.** *PLoS pathog* 2011, **7**(8):e1002166.
43. Weiner HL, Fields BN: **Neutralization of reovirus: the gene responsible for the neutralization antigen.** *J Exp Med* 1977, **146**(5):1305-1310.
44. Mohamed A, Johnston RN, Shmulevitz M: **Potential for Improving Potency and Specificity of Reovirus Oncolysis with Next-Generation Reovirus Variants.** *Viruses* 2015, **7**(12):6251-6278.
45. Liemann S, Chandran K, Baker TS, Nibert ML, Harrison SC: **Structure of the reovirus membrane-penetration protein, Mu1, in a complex with its protector protein, Sigma3.** *Cell* 2002, **108**(2):283-295.
46. Cross RK, Fields BN: **Reovirus-specific polypeptides: analysis using discontinuous gel electrophoresis.** *J Virol* 1976, **19**(1):162-173.
47. Lee PW, Hayes EC, Joklik WK: **Characterization of anti-reovirus immunoglobulins secreted by cloned hybridoma cell lines.** *Virology* 1981, **108**(1):134-146.
48. Smith RE, Zweerink HJ, Joklik WK: **Polypeptide components of virions, top component and cores of reovirus type 3.** *Virology* 1969, **39**(4):791-810.
49. Zweerink HJ, Joklik WK: **Studies on the intracellular synthesis of reovirus-specified proteins.** *Virology* 1970, **41**(3):501-518.
50. Nibert ML, Schiff LA, Fields BN: **Mammalian reoviruses contain a myristoylated structural protein.** *J Virol* 1991, **65**(4):1960-1967.
51. Nibert ML, Fields BN: **A carboxy-terminal fragment of protein mu 1/mu 1C is present in infectious subvirion particles of mammalian reoviruses and is proposed to have a role in penetration.** *J Virol* 1992, **66**(11):6408-6418.

52. Imani F, Jacobs BL: **Inhibitory activity for the interferon-induced protein kinase is associated with the reovirus serotype 1 sigma 3 protein.** *Proc Natl Acad Sci USA* 1988, **85**(21):7887-7891.
53. Hovanessian AG: **Interferon-induced and double-stranded RNA-activated enzymes: a specific protein kinase and 2',5'-oligoadenylate synthetases.** *J Interferon Res* 1991, **11**(4):199-205.
54. Smith JA, Schmechel SC, Williams BR, Silverman RH, Schiff LA: **Involvement of the interferon-regulated antiviral proteins PKR and RNase L in reovirus-induced shutoff of cellular translation.** *J Virol* 2005, **79**(4):2240-2250.
55. Markussen T, Dahle MK, Tengs T, Løvoll M, Finstad Ø, Wiik-Nielsen CR, Grove S, Lauksund S, Robertsen B, Rimstad E: **Sequence analysis of the genome of piscine orthoreovirus (PRV) associated with heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (*Salmo salar*).** *PloS One* 2013, **8**.
56. Busch LK, Rodriguez-Grille J, Casal JI, Martinez-Costas J, Benavente J: **Avian and mammalian reoviruses use different molecular mechanisms to synthesize their microNS isoforms.** *J Gen Virol* 2011, **92**(Pt 11):2566-2574.
57. Novoa RR, Calderita G, Arranz R, Fontana J, Granzow H, Risco C: **Virus factories: associations of cell organelles for viral replication and morphogenesis.** *Biol Cell* 2005, **97**(2):147-172.
58. Rhim JS, Jordan LE, Mayor HD: **Cytochemical, fluorescent-antibody and electron microscopic studies on the growth of reovirus (ECHO 10) in tissue culture.** *Virology* 1962, **17**:342-355.
59. Fernandez de Castro I, Zamora PF, Ooms L, Fernandez JJ, Lai CM, Mainou BA, Dermody TS, Risco C: **Reovirus forms neo-organelles for progeny particle assembly within reorganized cell membranes.** *mBio* 2014, **5**(1).
60. Finstad OW, Dahle MK, Lindholm TH, Nyman IB, Lovoll M, Wallace C, Olsen CM, Storset AK, Rimstad E: **Piscine orthoreovirus (PRV) infects Atlantic salmon erythrocytes.** *Vet Res* 2014, **45**:35.
61. Wessel O, Olsen CM, Rimstad E, Dahle MK: **Piscine orthoreovirus (PRV) replicates in Atlantic salmon (*Salmo salar* L.) erythrocytes ex vivo.** *Vet Res* 2015, **46**:26.
62. Dermody TS, Nibert ML, Bassel-Duby R, Fields BN: **A sigma 1 region important for hemagglutination by serotype 3 reovirus strains.** *J Virol* 1990, **64**(10):5173-5176.
63. Konopka-Anstadt JL, Mainou BA, Sutherland DM, Sekine Y, Strittmatter SM, Dermody TS: **The Nogo receptor NgR1 mediates infection by mammalian reovirus.** *Cell Host Microbe* 2014, **15**(6):681-691.
64. Morrison LA, Sidman RL, Fields BN: **Direct spread of reovirus from the intestinal lumen to the central nervous system through vagal autonomic nerve fibers.** *Proc Natl Acad Sci USA* 1991, **88**(9):3852-3856.
65. Kauffman RS, Wolf JL, Finberg R, Trier JS, Fields BN: **The sigma 1 protein determines the extent of spread of reovirus from the gastrointestinal tract of mice.** *Virology* 1983, **124**(2):403-410.
66. Amin AB, Mortensen L, Poppe T: **Histology atlas. Normal structure of salmonids.** Bodø: Akvapatologisk Laboratorium AS; 1992.
67. Morera D, Roher N, Ribas L, Balasch JC, Donate C, Callol A, Boltana S, Roberts S, Goetz G, Goetz FW *et al*: **RNA-Seq reveals an integrated immune response in nucleated erythrocytes.** *PloS One* 2011, **6**(10):e26998.
68. Morera D, MacKenzie SA: **Is there a direct role for erythrocytes in the immune response?** *Vet Res* 2011, **42**:89.

69. Dahle MK, Wessel O, Timmerhaus G, Nyman IB, Jorgensen SM, Rimstad E, Krasnov A: **Transcriptome analyses of Atlantic salmon (*Salmo salar* L.) erythrocytes infected with piscine orthoreovirus (PRV).** *Fish Shellfish Immunol* 2015, **45**(2):780-790.
70. Conroy DA: **Basic atlas of Atlantic salmon (*Salmo Salar* L.) blood cells.** Carrickfergus: Patterson Peddie Consulting Ltd; 2006.
71. Conroy DA: **Studies on the haematology of the Atlantic salmon (*Salmo Salar* L.).** In: *Diseases of fish. Volume 30*, edn. Edited by Mawdeley-Thomas LE. London: Academic Press; 1972: 101-127.
72. Passantino L, Altamura M, Cianciotta A, Jirillo F, Ribaud MR, Jirillo E, Passantino GF: **Maturation of fish erythrocytes coincides with changes in their morphology, enhanced ability to interact with *Candida albicans* and release of cytokine-like factors active upon autologous macrophages.** *Immunopharmacol Immunotoxicol* 2004, **26**(4):573-585.
73. Lewis JM, Klein G, Walsh PJ, Currie S: **Rainbow trout (*Oncorhynchus mykiss*) shift the age composition of circulating red blood cells towards a younger cohort when exposed to thermal stress.** *J Comp Physiol B* 2012, **182**(5):663-671.
74. Krasnov A, Timmerhaus G, Afanasyev S, Takle H, Jorgensen SM: **Induced erythropoiesis during acute anemia in Atlantic salmon: a transcriptomic survey.** *Gen Comp Endocrinol* 2013, **192**:181-190.
75. Secombes CJ, Ellis AE: **The immunology of teleosts.** In: *Fish pathology*. Edited by Roberts RJ. Oxford: Blackwell Publishing Ltd; 2012.
76. Kongtorp RT, Kjerstad A, Taksdal T, Guttvik A, Falk K: **Heart and skeletal muscle inflammation in Atlantic salmon, *Salmo salar* L: a new infectious disease.** *J Fish Dis* 2004, **27**.
77. Kongtorp RT, Taksdal T: **Studies with experimental transmission of heart and skeletal muscle inflammation in Atlantic salmon, *Salmo salar* L.** *J fish dis* 2009, **32**.
78. Løvoll M, Alarcon M, Jensen BB, Taksdal T, Kristoffersen AB, Tengs T: **Quantification of piscine reovirus (PRV) at different stages of Atlantic salmon *Salmo salar* production.** *Dis Aquat Org* 2012, **99**.
79. Hauge H, Dahle M, Moldal T, Thoen E, Gjevne A-G, Weli S, Alarcón M, Grove S: **Piscine orthoreovirus can infect and shed through the intestine in experimentally challenged Atlantic salmon (*Salmo salar* L.).** *Vet Res* 2016, **47**(1):57.
80. Maginnis MS, Forrest JC, Kopecky-Bromberg SA, Dickeson SK, Santoro SA, Zutter MM, Nemerow GR, Bergelson JM, Dermody TS: **Beta1 integrin mediates internalization of mammalian reovirus.** *J Virol* 2006, **80**(6):2760-2770.
81. Ehrlich M, Boll W, Van Oijen A, Hariharan R, Chandran K, Nibert ML, Kirchhausen T: **Endocytosis by random initiation and stabilization of clathrin-coated pits.** *Cell* 2004, **118**(5):591-605.
82. Mainou BA, Dermody TS: **Transport to late endosomes is required for efficient reovirus infection.** *J Virol* 2012, **86**(16):8346-8358.
83. Golden JW, Bahe JA, Lucas WT, Nibert ML, Schiff LA: **Cathepsin S supports acid-independent infection by some reoviruses.** *J Biol Chem* 2004, **279**(10):8547-8557.
84. Ebert DH, Deussing J, Peters C, Dermody TS: **Cathepsin L and cathepsin B mediate reovirus disassembly in murine fibroblast cells.** *J Biol Chem* 2002, **277**(27):24609-24617.

85. Sturzenbecker LJ, Nibert M, Furlong D, Fields BN: **Intracellular digestion of reovirus particles requires a low pH and is an essential step in the viral infectious cycle.** *J Virol* 1987, **61**(8):2351-2361.
86. Borsa J, Copps TP, Sargent MD, Long DG, Chapman JD: **New intermediate subviral particles in the in vitro uncoating of reovirus virions by chymotrypsin.** *J Virol* 1973, **11**(4):552-564.
87. Shatkin AJ, LaFiandra AJ: **Transcription by infectious subviral particles of reovirus.** *J Virol* 1972, **10**(4):698-706.
88. Mayor HD, Jamison RM, Jordan LE, Vanmitchell M: **Reoviruses. II. Structure and Composition of the Virion.** *J Bact* 1965, **89**:1548-1556.
89. Wilson GJ, Nason EL, Hardy CS, Ebert DH, Wetzel JD, Venkataram Prasad BV, Dermody TS: **A single mutation in the carboxy terminus of reovirus outer-capsid protein sigma 3 confers enhanced kinetics of sigma 3 proteolysis, resistance to inhibitors of viral disassembly, and alterations in sigma 3 structure.** *J Virol* 2002, **76**(19):9832-9843.
90. Borsa J, Morash BD, Sargent MD, Copps TP, Lievaart PA, Szekely JG: **Two modes of entry of reovirus particles into L cells.** *J gen virol* 1979, **45**(1):161-170.
91. Bass DM, Bodkin D, Dambrauskas R, Trier JS, Fields BN, Wolf JL: **Intraluminal proteolytic activation plays an important role in replication of type 1 reovirus in the intestines of neonatal mice.** *J Virol* 1990, **64**(4):1830-1833.
92. Schulz WL, Haj AK, Schiff LA: **Reovirus uses multiple endocytic pathways for cell entry.** *J Virol* 2012, **86**(23):12665-12675.
93. Chandran K, Farsetta DL, Nibert ML: **Strategy for nonenveloped virus entry: a hydrophobic conformer of the reovirus membrane penetration protein micro 1 mediates membrane disruption.** *J Virol* 2002, **76**(19):9920-9933.
94. Dryden KA, Wang G, Yeager M, Nibert ML, Coombs KM, Furlong DB, Fields BN, Baker TS: **Early steps in reovirus infection are associated with dramatic changes in supramolecular structure and protein conformation: analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction.** *J Cell Biol* 1993, **122**(5):1023-1041.
95. Zhang L, Chandran K, Nibert ML, Harrison SC: **Reovirus mu1 structural rearrangements that mediate membrane penetration.** *J Virol* 2006, **80**(24):12367-12376.
96. Madren JA, Sarkar P, Danthi P: **Cell entry-associated conformational changes in reovirus particles are controlled by host protease activity.** *J Virol* 2012, **86**(7):3466-3473.
97. Joklik WK: **Studies on the effect of chymotrypsin on reovirions.** *Virology* 1972, **49**(3):700-715.
98. Furuichi Y, Muthukrishnan S, Tomasz J, Shatkin AJ: **Mechanism of formation of reovirus mRNA 5'-terminal blocked and methylated sequence, m7GpppGmpC.** *J Biol Chem* 1976, **251**(16):5043-5053.
99. Banerjee AK, Shatkin AJ: **Transcription in vitro by reovirus-associated ribonucleic acid-dependent polymerase.** *J Virol* 1970, **6**(1):1-11.
100. Joklik WK: **Structure and function of the reovirus genome.** *Microbiol Rev* 1981, **45**(4):483-501.
101. Skehel JJ, Joklik WK: **Studies on the in vitro transcription of reovirus RNA catalyzed by reovirus cores.** *Virology* 1969, **39**(4):822-831.
102. Watanabe Y, Millward S, Graham AF: **Regulation of transcription of the Reovirus genome.** *J Mol Biol* 1968, **36**(1):107-123.

103. McCrae MA: **Terminal structure of reovirus RNAs.** *J Gen Virol* 1981, **55**(Pt 2):393-403.
104. Silverstein SC, Dales S: **The penetration of reovirus RNA and initiation of its genetic function in L-strain fibroblasts.** *J Cell Biol* 1968, **36**(1):197-230.
105. Nonoyama M, Millward S, Graham AF: **Control of transcription of the reovirus genome.** *Nucleic Acids Res* 1974, **1**(3):373-385.
106. Spandidos DA, Krystal G, Graham AF: **Regulated transcription of the genomes of defective virions and temperature-sensitive mutants of reovirus.** *J Virol* 1976, **18**(1):7-19.
107. Ito Y, Joklik WK: **Temperature-sensitive mutants of reovirus. I. Patterns of gene expression by mutants of groups C, D, and E.** *Virology* 1972, **50**(1):189-201.
108. Bartlett NM, Gillies SC, Bullivant S, Bellamy AR: **Electron microscopy study of reovirus reaction cores.** *J Virol* 1974, **14**(2):315-326.
109. Gillies S, Bullivant S, Bellamy AR: **Viral RNA polymerases: electron microscopy of reovirus reaction cores.** *Science* 1971, **174**(4010):694-696.
110. Furuichi Y, Morgan M, Muthukrishnan S, Shatkin AJ: **Reovirus messenger RNA contains a methylated, blocked 5'-terminal structure: m-7G(5')ppp(5')G-MpCp.** *Proc Natl Acad Sci USA* 1975, **72**(1):362-366.
111. Mangus DA, Evans MC, Jacobson A: **Poly(A)-binding proteins: multifunctional scaffolds for the post-transcriptional control of gene expression.** *Genome Biol* 2003, **4**(7):223.
112. Yan L, Zhang J, Guo H, Yan S, Chen Q, Zhang F, Fang Q: **Aquareovirus NS80 initiates efficient viral replication by retaining core proteins within replication-associated viral inclusion bodies.** *PloS One* 2015, **10**(5):e0126127.
113. Netherton C, Moffat K, Brooks E, Wileman T: **A guide to viral inclusions, membrane rearrangements, factories, and viroplasm produced during virus replication.** *Adv Virus Res* 2007, **70**:101-182.
114. Xu P, Miller SE, Joklik WK: **Generation of reovirus core-like particles in cells infected with hybrid vaccinia viruses that express genome segments L1, L2, L3, and S2.** *Virology* 1993, **197**(2):726-731.
115. Schonberg M, Silverstein SC, Levin DH, Acs G: **Asynchronous synthesis of the complementary strands of the reovirus genome.** *Proc Natl Acad Sci USA* 1971, **68**(2):505-508.
116. Tytell AA, Lampson GP, Field AK, Hilleman MR: **Inducers of interferon and host resistance. 3. Double-stranded RNA from reovirus type 3 virions (reo 3-RNA).** *Proc Natl Acad Sci USA* 1967, **58**(4):1719-1722.
117. Nibert ML: **Structure of mammalian orthoreovirus particles.** *Curr Top Microbiol Immunol* 1998, **233**(Pt 1):1-30.
118. Becker MM, Peters TR, Dermody TS: **Reovirus sigma NS and mu NS proteins form cytoplasmic inclusion structures in the absence of viral infection.** *J Virol* 2003, **77**(10):5948-5963.
119. de Castro IF, Volonte L, Risco C: **Virus factories: biogenesis and structural design.** *Cell Microbiol* 2013, **15**(1):24-34.
120. Broering TJ, Kim J, Miller CL, Piggott CD, Dinoso JB, Nibert ML, Parker JS: **Reovirus nonstructural protein muNS recruits viral core surface proteins and entering core particles to factory-like inclusions.** *J Virol* 2004, **78**(4):1882-1892.
121. Broering TJ, Parker JS, Joyce PL, Kim J, Nibert ML: **Mammalian reovirus nonstructural protein microNS forms large inclusions and colocalizes with reovirus microtubule-associated protein micro2 in transfected cells.** *J Virol* 2002, **76**(16):8285-8297.

122. Carroll K, Hastings C, Miller CL: **Amino acids 78 and 79 of Mammalian Orthoreovirus protein microNS are necessary for stress granule localization, core protein lambda 2 interaction, and de novo virus replication.** *Virology* 2014, **448**:133-145.
123. Miller CL, Arnold MM, Broering TJ, Hastings CE, Nibert ML: **Localization of mammalian orthoreovirus proteins to cytoplasmic factory-like structures via nonoverlapping regions of microNS.** *J Virol* 2010, **84**(2):867-882.
124. Broering TJ, Kim J, Miller CL, Piggott CD, Dinoso JB, Nibert ML, Parker JS: **Reovirus nonstructural protein mu NS recruits viral core surface proteins and entering core particles to factory-like inclusions.** *J Virol* 2004, **78**(4):1882-1892.
125. Broering TJ, Arnold MM, Miller CL, Hurt JA, Joyce PL, Nibert ML: **Carboxyl-proximal regions of reovirus nonstructural protein muNS necessary and sufficient for forming factory-like inclusions.** *J Virol* 2005, **79**(10):6194-6206.
126. Touris-Otero F, Cortez-San Martin M, Martinez-Costas J, Benavente J: **Avian reovirus morphogenesis occurs within viral factories and begins with the selective recruitment of sigmaNS and lambdaA to microNS inclusions.** *J Mol Biol* 2004, **341**(2):361-374.
127. Parker JS, Broering TJ, Kim J, Higgins DE, Nibert ML: **Reovirus core protein mu2 determines the filamentous morphology of viral inclusion bodies by interacting with and stabilizing microtubules.** *J Virol* 2002, **76**(9):4483-4496.
128. Tillotson L, Shatkin AJ: **Reovirus polypeptide sigma 3 and N-terminal myristoylation of polypeptide mu 1 are required for site-specific cleavage to mu 1C in transfected cells.** *J Virol* 1992, **66**(4):2180-2186.
129. Lai CM, Mainou BA, Kim KS, Dermody TS: **Directional release of reovirus from the apical surface of polarized endothelial cells.** *mBio* 2013, **4**(2):e00049-00013.
130. Kongtorp RT, Taksdal T, Lyngoy A: **Pathology of heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon *Salmo salar*.** *Dis Aquat Org* 2004, **59**(3):217-224.
131. van Dongen HM, Masoumi N, Witwer KW, Pegtel DM: **Extracellular Vesicles Exploit Viral Entry Routes for Cargo Delivery.** *Microbiol Mol Biol Rev* 2016, **80**(2):369-386.
132. Conde-Vancells J, Rodriguez-Suarez E, Embade N, Gil D, Matthiesen R, Valle M, Elortza F, Lu SC, Mato JM, Falcon-Perez JM: **Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes.** *J Proteome Res* 2008, **7**(12):5157-5166.
133. Chahar HS, Bao X, Casola A: **Exosomes and Their Role in the Life Cycle and Pathogenesis of RNA Viruses.** *Viruses* 2015, **7**(6):3204-3225.
134. Thery C, Zitvogel L, Amigorena S: **Exosomes: composition, biogenesis and function.** *Nat Rev Immunol* 2002, **2**(8):569-579.
135. Keller S, Sanderson MP, Stoeck A, Altevogt P: **Exosomes: from biogenesis and secretion to biological function.** *Immunol Lett* 2006, **107**(2):102-108.
136. van Niel G, Porto-Carreiro I, Simoes S, Raposo G: **Exosomes: a common pathway for a specialized function.** *J Biochem* 2006, **140**(1):13-21.
137. Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, van Eijndhoven MA, Hopmans ES, Lindenberg JL, de Gruijl TD, Wurdinger T, Middeldorp JM: **Functional delivery of viral miRNAs via exosomes.** *Proc Natl Acad Sci USA* 2010, **107**(14):6328-6333.
138. Narayanan A, Iordanskiy S, Das R, Van Duyne R, Santos S, Jaworski E, Guendel I, Sampey G, Dalby E, Iglesias-Ussel M *et al*: **Exosomes derived from HIV-1-**

- infected cells contain trans-activation response element RNA.** *J Biol Chem* 2013, **288**(27):20014-20033.
139. Qin Q, Hastings C, Miller CL: **Mammalian orthoreovirus particles induce and are recruited into stress granules at early times postinfection.** *J Virol* 2009, **83**(21):11090-11101.
 140. Anderson P, Kedersha N: **Stressful initiations.** *J Cell Sci* 2002, **115**(Pt 16):3227-3234.
 141. Qin Q: **Characterization of mammalian orthoreovirus (MRV) induced stress granules (SGs) and implications of eIF2 α phosphorylation on viral translation.** *Graduate Theses.* Iowa: Iowa State University; 2010.
 142. Dever TE: **Gene-specific regulation by general translation factors.** *Cell* 2002, **108**(4):545-556.
 143. Kimball SR: **Regulation of translation initiation by amino acids in eukaryotic cells.** *Prog Mol Subcell Biol* 2001, **26**:155-184.
 144. Qin Q, Carroll K, Hastings C, Miller CL: **Mammalian orthoreovirus escape from host translational shutoff correlates with stress granule disruption and is independent of eIF2 α phosphorylation and PKR.** *J Virol* 2011, **85**(17):8798-8810.
 145. Lloyd RE: **How do viruses interact with stress-associated RNA granules?** *PLoS Path* 2012, **8**(6):e1002741.
 146. Onomoto K, Jogi M, Yoo JS, Narita R, Morimoto S, Takemura A, Sambhara S, Kawaguchi A, Osari S, Nagata K *et al*: **Critical role of an antiviral stress granule containing RIG-I and PKR in viral detection and innate immunity.** *PloS One* 2012, **7**(8):e43031.
 147. Huismans H, Joklik WK: **Reovirus-coded polypeptides in infected cells: isolation of two native monomeric polypeptides with affinity for single-stranded and double-stranded RNA, respectively.** *Virology* 1976, **70**(2):411-424.
 148. Yue Z, Shatkin AJ: **Double-stranded RNA-dependent protein kinase (PKR) is regulated by reovirus structural proteins.** *Virology* 1997, **234**(2):364-371.
 149. Bergan V, Jagus R, Lauksund S, Kileng O, Robertsen B: **The Atlantic salmon Z-DNA binding protein kinase phosphorylates translation initiation factor 2 alpha and constitutes a unique orthologue to the mammalian dsRNA-activated protein kinase R.** *FEBS J* 2008, **275**(1):184-197.
 150. DeBiasi RL, Edelstein CL, Sherry B, Tyler KL: **Calpain inhibition protects against virus-induced apoptotic myocardial injury.** *J virol* 2001, **75**(1):351-361.
 151. O'Donnell SM, Hansberger MW, Connolly JL, Chappell JD, Watson MJ, Pierce JM, Wetzel JD, Han W, Barton ES, Forrest JC *et al*: **Organ-specific roles for transcription factor NF-kappaB in reovirus-induced apoptosis and disease.** *J Clin Invest* 2005, **115**(9):2341-2350.
 152. DeBiasi RL, Robinson BA, Sherry B, Bouchard R, Brown RD, Rizeq M, Long C, Tyler KL: **Caspase inhibition protects against reovirus-induced myocardial injury in vitro and in vivo.** *J Virol* 2004, **78**(20):11040-11050.
 153. Clarke P, DeBiasi RL, Goody R, Hoyt CC, Richardson-Burns S, Tyler KL: **Mechanisms of reovirus-induced cell death and tissue injury: role of apoptosis and virus-induced perturbation of host-cell signaling and transcription factor activation.** *Viral Immunol* 2005, **18**(1):89-115.
 154. Clarke P, Richardson-Burns SM, DeBiasi RL, Tyler KL: **Mechanisms of apoptosis during reovirus infection.** *Curr Top Microbiol Immunol* 2005, **289**:1-24.
 155. Sherry B, Blum MA: **Multiple viral core proteins are determinants of reovirus-induced acute myocarditis.** *J Virol* 1994, **68**(12):8461-8465.

156. Sherry B, Fields BN: **The reovirus M1 gene, encoding a viral core protein, is associated with the myocarditic phenotype of a reovirus variant.** *J Virol* 1989, **63**(11):4850-4856.
157. Yousaf MN, Koppang EO, Skjodt K, Kollner B, Hordvik I, Zou J, Secombes C, Powell MD: **Cardiac pathological changes of Atlantic salmon (*Salmo salar* L.) affected with heart and skeletal muscle inflammation (HSMI).** *Fish Shellfish Immunol* 2012, **33**(2):305-315.
158. Mikalsen AB, Haugland O, Rode M, Solbakk IT, Evensen O: **Atlantic salmon reovirus infection causes a CD8 T cell myocarditis in Atlantic salmon (*Salmo salar* L.).** *PloS One* 2012, **7**(6):e37269.
159. Dahle MK, Wessel O, Timmerhaus G, Nyman IB, Jorgensen SM, Rimstad E, Krasnov A: **Transcriptome analyses of Atlantic salmon (*Salmo salar* L.) erythrocytes infected with piscine orthoreovirus (PRV).** *Fish Shellfish Immunol* 2015, **45**(2):780-790.
160. Paun A, Pitha PM: **The innate antiviral response: new insights into a continuing story.** *Adv Virus Res* 2007, **69**:1-66.
161. Jacobs BL, Langland JO: **When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA.** *Virology* 1996, **219**(2):339-349.
162. Xagorari A, Chlichlia K: **Toll-like receptors and viruses: induction of innate antiviral immune responses.** *Open Microbiol J* 2008, **2**:49-59.
163. Chen SN, Zou PF, Nie P: **Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) in fish: current knowledge and future perspectives.** *Immunology* 2017, **151**(1):16-25.
164. Takeuchi O, Akira S: **Innate immunity to virus infection.** *Immunol Rev* 2009, **227**(1):75-86.
165. Samuel CE: **Antiviral actions of interferons.** *Clin Microbiol Rev* 2001, **14**(4):778-809, table of contents.
166. Heim MH: **The Jak-STAT pathway: cytokine signalling from the receptor to the nucleus.** *J Recept Signal Transduct Res* 1999, **19**(1-4):75-120.
167. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD: **How cells respond to interferons.** *Annu Rev Biochem* 1998, **67**:227-264.
168. Verrier ER, Langevin C, Benmansour A, Boudinot P: **Early antiviral response and virus-induced genes in fish.** *Dev comp immunol* 2011, **35**(12):1204-1214.
169. Zinzula L, Tramontano E: **Strategies of highly pathogenic RNA viruses to block dsRNA detection by RIG-I-like receptors: hide, mask, hit.** *Antiviral Res* 2013, **100**(3):615-635.
170. Holm GH, Zurney J, Tumilasci V, Leveille S, Danthi P, Hiscott J, Sherry B, Dermody TS: **Retinoic acid-inducible gene-I and interferon-beta promoter stimulator-1 augment proapoptotic responses following mammalian reovirus infection via interferon regulatory factor-3.** *J Biol Chem* 2007, **282**(30):21953-21961.
171. Skup D, Millward S: **Reovirus-induced modification of cap-dependent translation in infected L cells.** *Proc Natl Acad Sci USA* 1980, **77**(1):152-156.
172. Sharpe AH, Fields BN: **Reovirus inhibition of cellular RNA and protein synthesis: role of the S4 gene.** *Virology* 1982, **122**(2):381-391.
173. Cai R, Williams BR: **Mutations in the double-stranded RNA-activated protein kinase insert region that uncouple catalysis from eIF2alpha binding.** *J Biol Chem* 1998, **273**(18):11274-11280.

174. Henderson DR, Joklik WK: **The mechanism of interferon induction by UV-irradiated reovirus.** *Virology* 1978, **91**(2):389-406.
175. Lai MH, Joklik WK: **The induction of interferon by temperature-sensitive mutants of reovirus, UV-irradiated reovirus, and subviral reovirus particles.** *Virology* 1973, **51**(1):191-204.
176. Sherry B, Torres J, Blum MA: **Reovirus induction of and sensitivity to beta interferon in cardiac myocyte cultures correlate with induction of myocarditis and are determined by viral core proteins.** *J Virol* 1998, **72**(2):1314-1323.
177. Rosaeg MV, Lund M, Nyman IB, Markussen T, Aspehaug V, Sindre H, Dahle MK, Rimstad E: **Immunological interactions between Piscine orthoreovirus and Salmonid alphavirus infections in Atlantic salmon.** *Fish Shellfish Immunol* 2017, **64**:308-319.
178. Garver KA, Johnson SC, Polinski MP, Bradshaw JC, Marty GD, Snyman HN, Morrison DB, Richard J: **Piscine Orthoreovirus from Western North America Is Transmissible to Atlantic Salmon and Sockeye Salmon but Fails to Cause Heart and Skeletal Muscle Inflammation.** *PloS One* 2016, **11**(1):e0146229.
179. Mutoloki S, Jørgensen JB, Evensen Ø: **The Adaptive Immune Response in Fish.** In: *Fish Vaccination.* John Wiley & Sons, Ltd; 2014: 104-115.
180. Munang'andu HM, Fredriksen BN, Mutoloki S, Dalmo RA, Evensen Ø: **Antigen dose and humoral immune response correspond with protection for inactivated infectious pancreatic necrosis virus vaccines in Atlantic salmon (*Salmo salar* L).** *Vet Res* 2013, **44**(1):7.
181. Graham DA, Rowley HR, Frost P: **Cross-neutralization studies with salmonid alphavirus subtype 1-6 strains: results with sera from experimental studies and natural infections.** *J Fish Dis* 2014, **37**(8):683-691.
182. Jorgensen SM, Afanasyev S, Krasnov A: **Gene expression analyses in Atlantic salmon challenged with infectious salmon anemia virus reveal differences between individuals with early, intermediate and late mortality.** *BMC genomics* 2008, **9**:179.
183. Teige LH, Lund M, Haatveit HM, Rosaeg MV, Wessel O, Dahle MK, Storset AK: **A bead based multiplex immunoassay detects Piscine orthoreovirus specific antibodies in Atlantic salmon (*Salmo salar*).** *Fish Shellfish Immunol* 2017, **63**:491-499.
184. Johansen LH, Thim HL, Jorgensen SM, Afanasyev S, Strandskog G, Taksdal T, Fremmerlid K, McLoughlin M, Jorgensen JB, Krasnov A: **Comparison of transcriptomic responses to pancreas disease (PD) and heart and skeletal muscle inflammation (HSMI) in heart of Atlantic salmon (*Salmo salar* L).** *Fish Shellfish Immunol* 2015, **46**(2):612-623.
185. Palacios G, Løvoll M, Tengs T, Hornig M, Hutchison S, Hui J, Kongtorp RT, Savji N, Bussetti AV, Solovyov A *et al*: **Heart and skeletal muscle inflammation of farmed salmon is associated with infection with a novel reovirus.** *PloS One* 2010, **5**.
186. Meyers TR: **First report of erythrocytic inclusion body syndrome (EIBS) in chinook salmon *Oncorhynchus tshawytscha* in Alaska, USA.** *Dis Aquat Org* 2007, **76**(2):169-172.
187. Rodger HD: **Erythrocytic inclusion body syndrome virus in wild Atlantic salmon, *Salmo salar* L.** *J Fish Dis* 2007, **30**(7):411-418.
188. Leek SL: **Viral Erythrocytic Inclusion Body Syndrome (EIBS) Occurring in Juvenile Spring Chinook Salmon (*Oncorhynchus tshawytscha*) Reared in Freshwater.** *Can J Fish Aquat Sci* 1987, **44**(3):685-688.

189. Okamoto N, Takahashi K, Maita M, Rohovec JS, Ikeda Y: **Erythrocytic Inclusion Body Syndrome : Susceptibility of Selected Sizes of Coho Salmon and of Several Other Species of Salmonid Fish.** *Fish Path* 1992, **27**(3):153-156.
190. Wiik-Nielsen CR, Ski PM, Aunsmo A, Lovoll M: **Prevalence of viral RNA from piscine reovirus and piscine myocarditis virus in Atlantic salmon, *Salmo salar* L., broodfish and progeny.** *J Fish Dis* 2012, **35**(2):169-171.
191. Marty GD, Morrison DB, Bidulka J, Joseph T, Siah A: **Piscine reovirus in wild and farmed salmonids in British Columbia, Canada: 1974–2013.** *J Fish Dis* 2014, **38**(8):713-728.
192. Garseth Å, Fritsvold C, Opheim M, Skjerve E, Biering E: **Piscine reovirus (PRV) in wild Atlantic salmon, *Salmo salar* L., and sea-trout, *Salmo trutta* L., in Norway.** *J Fish Dis* 2013, **36**.
193. Kibenge MJ, Iwamoto T, Wang Y, Morton A, Godoy MG, Kibenge FS: **Whole-genome analysis of piscine reovirus (PRV) shows PRV represents a new genus in family Reoviridae and its genome segment S1 sequences group it into two separate sub-genotypes.** *Virol J* 2013, **10**:230.
194. Di Cicco E, Ferguson HW, Schulze AD, Kaukinen KH, Li S, Vanderstichel R, Wessel O, Rimstad E, Gardner IA, Hammell KL *et al*: **Heart and skeletal muscle inflammation (HSMI) disease diagnosed on a British Columbia salmon farm through a longitudinal farm study.** *PLoS One* 2017, **12**(2):e0171471.
195. Garver KA, Johnson SC, Polinski MP, Bradshaw JC, Marty GD, Snyman HN, Morrison DB, Richard J: **Piscine orthoreovirus from western North America is transmissible to Atlantic salmon and Sockeye salmon but fails to cause heart and skeletal muscle inflammation.** *PLoS One* 2016, **11**(1).
196. Lovoll M, Alarcon M, Bang Jensen B, Taksdal T, Kristoffersen AB, Tengs T: **Quantification of Piscine reovirus (PRV) at different stages of Atlantic salmon *Salmo salar* production.** *Dis Aquat Org* 2012, **99**(1):7-12.
197. Kristoffersen AB, Bang Jensen B, Jansen PA: **Risk mapping of heart and skeletal muscle inflammation in salmon farming.** *Prev Vet Med* 2013, **109**(1-2):136-143.
198. Løvoll M, Wiik-Nielsen J, Grove S, Wiik-Nielsen CR, Kristoffersen AB, Faller R, Poppe T, Jung J, Pedomallu CS, Nederbragt AJ *et al*: **A novel totivirus and piscine reovirus (PRV) in Atlantic salmon (*Salmo salar*) with cardiomyopathy syndrome (CMS).** *Virol J* 2010, **7**.
199. Haugland O, Mikalsen AB, Nilsen P, Lindmo K, Thu BJ, Eliassen TM, Roos N, Rode M, Evensen O: **Cardiomyopathy syndrome of atlantic salmon (*Salmo salar* L.) is caused by a double-stranded RNA virus of the Totiviridae family.** *J Virol* 2011, **85**(11):5275-5286.
200. Yousaf MN, Koppang EO, Skjodt K, Hordvik I, Zou J, Secombes C, Powell MD: **Comparative cardiac pathological changes of Atlantic salmon (*Salmo salar* L.) affected with heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and pancreas disease (PD).** *Vet Immunol Immunopathol* 2013, **151**(1-2):49-62.
201. Taksdal T, Olsen AB, Bjerkas I, Hjortaas MJ, Dannevig BH, Graham DA, McLoughlin MF: **Pancreas disease in farmed Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), in Norway.** *J Fish Dis* 2007, **30**(9):545-558.
202. Poppe T, Ferguson HW: **Cardiovascular system.** In: *Systemic pathology of fish: A text and atlas of normal tissue response in teleosts, and their responses in disease.* Edited by Ferguson HW. London: Scotian Press; 2006: 141-167.

203. Mørkøre T, Heia K: **Black spots in salmon fillet - extent and methods of measurement.** *Norsk Fiskeoppdrett* 2012(3):50-53.
204. Koppang EO, Haugarvoll E, Hordvik I, Aune L, Poppe TT: **Vaccine-associated granulomatous inflammation and melanin accumulation in Atlantic salmon, *Salmo salar* L., white muscle.** *J Fish Dis* 2005, **28**(1):13-22.
205. Larsen HA, Austbo L, Morkore T, Thorsen J, Hordvik I, Fischer U, Jirillo E, Rimstad E, Koppang EO: **Pigment-producing granulomatous myopathy in Atlantic salmon: a novel inflammatory response.** *Fish Shellfish Immunol* 2012, **33**(2):277-285.
206. Haugarvoll E, Thorsen J, Laane M, Huang Q, Koppang EO: **Melanogenesis and evidence for melanosome transport to the plasma membrane in a CD83 teleost leukocyte cell line.** *Pigment Cell Res* 2006, **19**(3):214-225.
207. Agius C, Roberts RJ: **Melano-macrophage centres and their role in fish pathology.** *J Fish Dis* 2003, **26**(9):499-509.
208. Larsen HA, Austbo L, Nodtvedt A, Fraser TW, Rimstad E, Fjellidal PG, Hansen T, Koppang EO: **The effect of vaccination, ploidy and smolt production regime on pathological melanin depositions in muscle tissue of Atlantic salmon, *Salmo salar* L.** *J Fish Dis* 2014, **37**(4):327-340.
209. Waagbø R: **Reducing production-related diseases in farmed fish.** In: *Improving Farmed Fish Quality and Safety.* Edited by Lie Ø: Woodhead Publishing; 2008: 363-398.
210. Braithwaite VA, Boulcott P: **Pain perception, aversion and fear in fish.** *Dis Aquat Org* 2007, **75**(2):131-138.
211. Braithwaite VA, Ebbesson LO: **Pain and stress responses in farmed fish.** *Rev Sci Tech* 2014, **33**(1):245-253.
212. Ferguson HW, Kongtorp RT, Taksdal T, Graham D, Falk K: **An outbreak of disease resembling heart and skeletal muscle inflammation in Scottish farmed salmon, *Salmo salar* L., with observations on myocardial regeneration.** *J Fish Dis* 2005, **28**(2):119-123.
213. Godoy MG, Kibenge MJ, Wang Y, Suarez R, Leiva C, Vallejos F, Kibenge FS: **First description of clinical presentation of piscine orthoreovirus (PRV) infections in salmonid aquaculture in Chile and identification of a second genotype (Genotype II) of PRV.** *Virol J* 2016, **13**(1):98.
214. Ane H, Thomassen MS, Takle H, Terjesen BF, Grammes F, Oehme M, Refstie S, Sigholt T, Berge RK, Rorvik KA: **Increased survival by feeding tetradecylthioacetic acid during a natural outbreak of heart and skeletal muscle inflammation in S0 Atlantic salmon, *Salmo salar* L.** *J Fish Dis* 2009, **32**(11):953-961.
215. Gomez-Casado E, Estepa A, Coll JM: **A comparative review on European-farmed finfish RNA viruses and their vaccines.** *Vaccine* 2011, **29**(15):2657-2671.
216. Abdullah A, Olsen CM, Hodneland K, Rimstad E: **A polyprotein-expressing salmonid alphavirus replicon induces modest protection in atlantic salmon (*Salmo salar*) against infectious pancreatic necrosis.** *Viruses* 2015, **7**(1):252-267.
217. Evensen O, Leong JA: **DNA vaccines against viral diseases of farmed fish.** *Fish Shellfish Immunol* 2013, **35**(6):1751-1758.
218. Karlsen M, Villoing S, Rimstad E, Nylund A: **Characterization of untranslated regions of the salmonid alphavirus 3 (SAV3) genome and construction of a SAV3 based replicon.** *Virol J* 2009, **6**:173.
219. Karlsen M, Villoing S, Ottem KF, Rimstad E, Nylund A: **Development of infectious cDNA clones of Salmonid alphavirus subtype 3.** *BMC Res* 2010, **3**:241.

220. Wolf A, Hodneland K, Frost P, Braaen S, Rimstad E: **A hemagglutinin-esterase-expressing salmonid alphavirus replicon protects Atlantic salmon (*Salmo salar*) against infectious salmon anemia (ISA).** *Vaccine* 2013, **31**(4):661-669.
221. Hikke MC, Braaen S, Villoing S, Hodneland K, Geertsema C, Verhagen L, Frost P, Vlak JM, Rimstad E, Pijlman GP: **Salmonid alphavirus glycoprotein E2 requires low temperature and E1 for virion formation and induction of protective immunity.** *Vaccine* 2014, **32**(47):6206-6212.
222. Rajesh Kumar S, Ishaq Ahamed VP, Sarathi M, Nazeer Basha A, Sahul Hameed AS: **Immunological responses of *Penaeus monodon* to DNA vaccine and its efficacy to protect shrimp against white spot syndrome virus (WSSV).** *Fish Shellfish Immunol* 2008, **24**(4):467-478.
223. Rajeshkumar S, Venkatesan C, Sarathi M, Sarathbabu V, Thomas J, Anver Basha K, Sahul Hameed AS: **Oral delivery of DNA construct using chitosan nanoparticles to protect the shrimp from white spot syndrome virus (WSSV).** *Fish Shellfish Immunol* 2009, **26**(3):429-437.
224. Kutzler MA, Weiner DB: **DNA vaccines: ready for prime time?** *Nat Rev Genet* 2008, **9**(10):776-788.
225. Saloni K, Simard N, Harland R, Ulmer JB: **The road to licensure of a DNA vaccine.** *Curr Opin Investig Drugs* 2007, **8**(8):635-641.
226. Tyler KL, Mann MA, Fields BN, Virgin HW: **Protective anti-reovirus monoclonal antibodies and their effects on viral pathogenesis.** *J Virol* 1993, **67**(6):3446-3453.
227. Dalet A, Gatti E, Pierre P: **Integration of PKR-dependent translation inhibition with innate immunity is required for a coordinated anti-viral response.** *FEBS Lett* 2015, **589**(14):1539-1545.
228. Durfee LA, Lyon N, Seo K, Huibregtse JM: **The ISG15 conjugation system broadly targets newly synthesized proteins: implications for the antiviral function of ISG15.** *Mol Cell* 2010, **38**(5):722-732.
229. McCutcheon AM, Broering TJ, Nibert ML: **Mammalian reovirus M3 gene sequences and conservation of coiled-coil motifs near the carboxyl terminus of the microNS protein.** *Virology* 1999, **264**(1):16-24.
230. Wiener JR, Bartlett JA, Joklik WK: **The sequences of reovirus serotype 3 genome segments M1 and M3 encoding the minor protein mu 2 and the major nonstructural protein mu NS, respectively.** *Virology* 1989, **169**(2):293-304.
231. Nibert ML, Odegard AL, Agosto MA, Chandran K, Schiff LA: **Putative autocleavage of reovirus mu1 protein in concert with outer-capsid disassembly and activation for membrane permeabilization.** *J Mol Biol* 2005, **345**(3):461-474.
232. Rayner JO, Dryga SA, Kamrud KI: **Alphavirus vectors and vaccination.** *Rev Med Virol* 2002, **12**(5):279-296.
233. Hornstein BD, Roman D, Arevalo-Soliz LM, Engevik MA, Zechiedrich L: **Effects of Circular DNA Length on Transfection Efficiency by Electroporation into HeLa Cells.** *PLoS One* 2016, **11**(12):e0167537.
234. Leitner WW, Hwang LN, deVeer MJ, Zhou A, Silverman RH, Williams BR, Dubensky TW, Ying H, Restifo NP: **Alphavirus-based DNA vaccine breaks immunological tolerance by activating innate antiviral pathways.** *Nat Med* 2003, **9**(1):33-39.
235. Knudsen ML, Mbewe-Mvula A, Rosario M, Johansson DX, Kakoulidou M, Bridgeman A, Reyes-Sandoval A, Nicosia A, Ljungberg K, Hanke T *et al*: **Superior induction of T cell responses to conserved HIV-1 regions by electroporated**

- alphavirus replicon DNA compared to that with conventional plasmid DNA vaccine.** *J Virol* 2012, **86**(8):4082-4090.
236. Chen Z, Sahashi Y, Matsuo K, Asanuma H, Takahashi H, Iwasaki T, Suzuki Y, Aizawa C, Kurata T, Tamura S: **Comparison of the ability of viral protein-expressing plasmid DNAs to protect against influenza.** *Vaccine* 1998, **16**(16):1544-1549.
237. Johnston JA, Ward CL, Kopito RR: **Aggresomes: a cellular response to misfolded proteins.** *J Cell Biol* 1998, **143**(7):1883-1898.
238. Wiik-Nielsen J, Alarcon M, Jensen BB, Haugland O, Mikalsen AB: **Viral co-infections in farmed Atlantic salmon, *Salmo salar* L., displaying myocarditis.** *J Fish Dis* 2016, **39**(12):1495-1507.
239. Lund M, Rosaeg MV, Krasnov A, Timmerhaus G, Nyman IB, Aspehaug V, Rimstad E, Dahle MK: **Experimental Piscine orthoreovirus infection mediates protection against pancreas disease in Atlantic salmon (*Salmo salar*).** *Vet Res* 2016, **47**(1):107.

9. Scientific papers I – III

I

RESEARCH ARTICLE

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The non-structural protein μ NS of piscine orthoreovirus (PRV) forms viral factory-like structures

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Abstract

Piscine orthoreovirus (PRV) is associated with heart- and skeletal muscle inflammation in farmed Atlantic salmon. The virus is ubiquitous and found in both farmed and wild salmonid fish. It belongs to the family *Reoviridae*, closely related to the genus *Orthoreovirus*. The PRV genome comprises ten double-stranded RNA segments encoding at least eight structural and two non-structural proteins. Erythrocytes are the major target cells for PRV. Infected erythrocytes contain globular inclusions resembling viral factories; the putative site of viral replication. For the mammalian reovirus (MRV), the non-structural protein μ NS is the primary organizer in factory formation. The analogous PRV protein was the focus of the present study. The subcellular location of PRV μ NS and its co-localization with the PRV σ NS, μ 2 and λ 1 proteins was investigated. We demonstrated that PRV μ NS forms dense globular cytoplasmic inclusions in transfected fish cells, resembling the viral factories of MRV. In co-transfection experiments with μ NS, the σ NS, μ 2 and λ 1 proteins were recruited to the globular structures. The ability of μ NS to recruit other PRV proteins into globular inclusions indicates that it is the main viral protein involved in viral factory formation and pivotal in early steps of viral assembly.

Introduction

Piscine orthoreovirus (PRV) is a member of the family *Reoviridae*. The virus is associated with heart and skeletal muscle inflammation (HSMI), an important emerging disease in the intensive farming of Atlantic salmon (*Salmo salar*) [1, 2]. HSMI is mainly observed during the seawater grow-out phase and there is often a prolonged disease development [3]. The cumulative mortality varies from negligible to 20%, while the morbidity is almost 100% in affected cages [3]. PRV seems to be ubiquitous in Norwegian salmon farms [4]. Fish kept at high stocking density with frequent handling experience a stressful environment that may result in immunosuppression and a greater disease burden, thus facilitating the rapid spread of pathogens [5]. PRV has also been detected in

wild salmon, but no lesions consistent with HSMI have been discovered in the wild population [6].

Phylogenetic analysis indicates that PRV branches off the common root of the genera *Orthoreovirus* and *Aquareovirus*, but most closely related to the orthoreoviruses [7, 8]. PRV differs from other orthoreoviruses like mammalian reoviruses (MRVs) and avian reoviruses (ARVs) in the ability to infect salmonid fish species at low temperatures, and in the preference for erythrocytes as one of the main target cells. The genome of PRV comprises ten double-stranded RNA (dsRNA) segments distributed in the classical orthoreoviral groups of three large, three medium and four small segments [1, 8, 9]. Currently, the PRV genome has been found to encode at least ten primary translation products. However, there is only a limited number of functional studies concerning the different proteins expressed by this virus [10, 11]. Based upon sequence homology to MRV, and the presence of conserved structures and motifs, eight of the deduced translation products are assumed structural components forming the orthoreovirus particle with an

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inner core and an outer capsid, while two of the translation products are non-structural proteins [8, 12].

A common feature for the non-structural proteins of reoviruses is their ability to form viral factories [13, 14]. Viral factories, also known as viroplasm or viral replication centers, are intracellular compartments for replication, packaging and assembly of viral particles [13, 15]. Several RNA and DNA viruses have been reported to induce these specialized membranous compartments within the cytoplasm of infected cells [16–18]. They commonly form as invaginations in a variety of organelles such as mitochondria, endoplasmic reticulum, lysosomes, peroxisomes, Golgi apparatus or chloroplasts [18, 19]. The factory scaffold facilitates spatial coordination of viral genome replication and assembly with the use of cell resources [18]. The viral factory inclusions seen during MRV infection consist of viral dsRNA, viral proteins, partially and fully assembled viral particles, microtubules and thinner filaments suggested to be intermediate structures [20]. Although organization of viral factories varies between different virus families, several fundamental similarities exist. Viruses utilize cellular biosynthetic pathways for their morphogenesis and propagation, and use a variety of mechanisms to avoid being wiped out by the cellular antiviral response [13, 21]. In the viral factories the viral pathogen-associated molecular patterns are shielded from inducing the activation of cellular innate responses [19].

Erythrocytes are major target cells for PRV, and in infected erythrocytes globular inclusions are formed and contain both PRV protein and dsRNA [22, 23]. The inclusions resemble the globular viral factories seen in MRV type 3 Dearing (T3D) prototype strain infected cells [19, 22]. Furthermore, the PRV inclusions contain reovirus-like particles as observed by transmission electron microscopy (TEM) [22]. This suggests that PRV, like MRV, forms viral factories in infected cells.

MRV μ NS is the scaffolding protein that organizes viral factories during MRV infection [24]. Comparison of the PRV μ NS amino acid sequence with the homologous proteins from MRV and ARV has revealed a very low sequence identity of only 17%, however, partially conserved motifs are present [8]. The latter includes a C-terminal motif shown for MRV μ NS to be required for the recruitment of clathrin to viral factories [8, 25]. Furthermore, predictions of MRV and ARV μ NS show two α -helical coils in their C-terminal region required for inclusion formation [26–29]. A high α -helical content in the C-terminal region is also predicted for the PRV μ NS, but coiled coil motifs are predicted with significantly lower probability than for MRV and ARV [8]. In addition, MRV and ARV have both been shown to produce two protein products from gene segment M3 [8,

30]. Whereas μ NS represents the full-length isoform, a second in-frame AUG (Met₄₁) in the MRV protein represents the translational start site for the second isoform μ NSC. In the ARV protein, post-translational cleavage near the N-terminal region creates μ NSN [8, 30]. In PRV M3, only one open reading frame (ORF) has been identified encoding the μ NS protein [8].

We hypothesized that the μ NS of PRV is an organization center in the assembly of progeny virus particles. The aim in this study was to examine the localization of PRV μ NS and its ability to interact with other PRV proteins in transfected cells.

Materials and methods

Cells

EPC cells (ATCC CRL-2872, *Epithelioma papulosum cyprini*) and CHSE-214 cells (ATCC CRL-1681, *Chinook salmon embryo*) were cultivated in Leibovitz-15 medium (L15, Life Technologies, Paisley, Scotland, UK) supplemented with 10% heat inactivated fetal bovine serum (FBS, Life technologies), 2 mM L-glutamine, 0.04 mM mercaptoethanol and 0.05 mg/mL gentamycin-sulphate (Life Technologies).

Computer analyses

Multiple sequence alignments were performed using AlignX (Vector NTI Advance™ 11, Invitrogen, Carlsbad, CA, USA) and protein secondary structure predictions using PSIPRED v3.0. The presence of putative nuclear localization signals (NLS) in PRV μ 2 was investigated using PSORTII, PredictProtein [31] and NLS mapper. The GenBank accession numbers for the PRV μ NS, σ NS, λ 1 and μ 2 coding sequences of the present study are KR337478, KR337481, KR337475 and KR337476, respectively.

Plasmid constructs

Total RNA was isolated from homogenized tissue from a natural outbreak of HSMI in Atlantic salmon (MH-050607) as previously described [8]. RNA was denatured at 95 °C for 5 min and transcribed into cDNA using SuperScript® III Reverse Transcriptase (RT) (Invitrogen) and Random Primers (Invitrogen) according to the manufacturer's protocol. PfuUltra II Fusion HS DNA polymerase (Agilent, Santa Clara, CA, USA) was used to amplify the ORFs of μ NS, σ NS, μ 2 and λ 1. The primers contained the sequences encoding flag-tag, myc-tag or HA-tag for protein recognition by antibodies [32]. Primer sequences are shown in Table 1. For both the full-length μ NS and σ NS constructs, a pair of expression vectors was made encoding proteins tagged in either the C-terminus or the N-terminus; pcDNA3.1- μ NS-N-FLAG, pcDNA3.1- μ NS-C-FLAG, pcDNA3.1- σ NS-N-MYC and

Table 1 Expression plasmids.

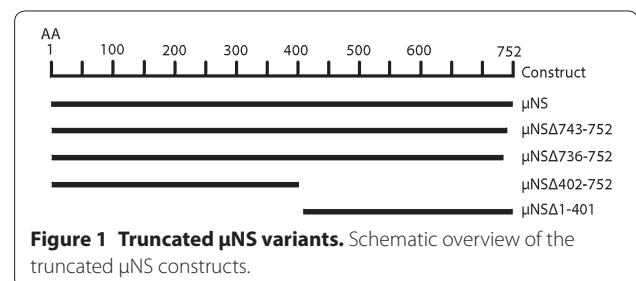
Plasmid name	Primer	Sequence (5' → 3')
pcDNA3.1-μNS-N-FLAG	Forward	GCCGCTCGAGTCTAGAGCCACC ATG GACTACAAAGACGATGACGACAAGATGGCTGAATCAATTACTTTTG
	Reverse	AAACGGGCCCTCTAGATCAGCCACGTAGCACATTATTAC
pcDNA3.1-μNS-C-FLAG	Forward	GCCGCTCGAGTCTAGAGCCACC ATG CGCAAGCTGGACTTGGTTGCA
	Reverse	AAACGGGCCCTCTAGATCACTTGTCTGTCATCGTCTTTGTAGTCGCCACGTAGCACATTATTACCGCC
pcDNA3.1-σNS-N-MYC	Forward	GCCGCTCGAGTCTAGAGCCACC ATG GAACAAAACTCATCTCAGAAGAGGATCTGATGTGCAACTTTGATCTTGG
	Reverse	AAACGGGCCCTCTAGACTAACAAAACATGGCCATGA
pcDNA3.1-σNS-C-MYC	Forward	GCCGCTCGAGTCTAGAGCCACC ATG TGCAACTTTGATCTTGG
	Reverse	AAACGGGCCCTCTAGACTACAGATCCTCTCTGAGATGAGTTTTGTTCACAAAACATGGCCATGATGC
pcDNA3.1-μ2-C-HA	Forward	GGCGGCCGCTCGAGTCTAGAA ATG CCTATCATAAACCTGCC
	Reverse	GTTTAAACGGGCCCTCTAGAAAGCGTAATCTGGAACATCGATGGGTACTIONACCAGCTGTAGACCACC
pcDNA3.1-λ1-N-HA	Forward	CGCTCGAGTCTAGAGCCACC ATG TACCCATACGATGTTCCAGATTACGCTATGGAGCGACTTAAGAGGAAAG
	Reverse	AAACGGGCCCTCTAGATTAGTTGAGTACAGGATGAG
pcDNA3.1-μNSΔ743-752	Forward	GCCGCTCGAGTCTAGAGCCACC ATG GACTACAAAGACGATGACGACAAGATGGCTGAATCAATTACTTTTG
	Reverse	AAACGGGCCCTCTAGATCACCAGTCATCTGAGCCACCAAA
pcDNA3.1-μNSΔ736-752	Forward	GCCGCTCGAGTCTAGAGCCACC ATG GACTACAAAGACGATGACGACAAGATGGCTGAATCAATTACTTTTG
	Reverse	AAACGGGCCCTCTAGATCAGTCGATGATTTTTGGAAACTC
pcDNA3.1-μNSΔ1-401	Forward	GCCGCTCGAGTCTAGAGCCACC ATG CCAACCACCTGGTATTCAAC
	Reverse	AAACGGGCCCTCTAGATCACTTGTCTGTCATCGTCTTTGTAGTCGCCACGTAGCACATTATTACCGCC
pcDNA3.1-μNSΔ402-752	Forward	GCCGCTCGAGTCTAGAGCCACC ATG GACTACAAAGACGATGACGACAAGATGGCTGAATCAATTACTTTTG
	Reverse	AAACGGGCCCTCTAGATCATGTGTGTCAGGGAATAGTGCAT

Primers used in generating the constructs encoding PRV μNS (M3), σNS (S3), μ2 (M1) and λ1 (L3) and truncated versions of μNS. Start codons are marked in bold and epitope tags in italic.

pcDNA3.1-σNS-C-MYC. For μ2, the tag was added only C-terminally and for λ1 only N-terminally, pcDNA3.1-μ2-C-MYC and pcDNA3.1-λ1-N-HA, respectively. Four truncated forms of the μNS protein with flag-tags C- or N-terminally depending on the truncation were also generated to determine sequence regions in PRV μNS involved in formation of viral factories during infection, pcDNA3.1-μNSΔ1-401, pcDNA3.1-μNSΔ402-752, pcDNA3.1-μNSΔ736-752 and pcDNA3.1-μNSΔ743-752 (Figure 1). In-fusion HD Cloning Kit (Clontech Laboratories, Mountain View, CA, USA) was used to clone PCR products into the XbaI restriction site of the eukaryotic expression vector pcDNA3.1(+) (Invitrogen). Sanger sequencing (GATC Biotech AG, Konstanz, Germany) verified all sequences. A pcDNA3.1 construct expressing the protein encoded by infectious salmon anemia virus (ISAV) segment 8 open reading frame 2 (S8ORF2) protein [33] was used as a control during transfections, immunoprecipitation and western blotting.

Transfections of fish cells

EPC and CHSE cells were seeded on gelatin embedded cover slips (12 mm) with pre-equilibrated L-15 growth medium at a density of 1.5×10^4 cells in a 24-well plate 24 h prior to transfection. Plasmids were transfected using Lipofectamine LTX reagent (Life Technologies)



according to the manufacturer's instructions. In brief, 2 μL lipofectamine was mixed with 0.5 μg plasmid and 0.5 μL PLUS reagent, and diluted in a total of 100 μL Opti-MEM (Life Technologies). After 5 min of incubation, the mixture was added to the cells and incubated at 20 °C for 48 h. When co-transfections were performed, a total of 0.4 μg of each plasmid were used and the amount of PLUS reagent was increased to 0.8 μL.

Immunofluorescence microscopy

Transfected EPC and CHSE cells were fixed and stained using an intracellular Fixation and Permeabilization Buffer (eBioscience, San Diego, CA, USA). The cells were washed in Dulbecco's PBS (DPBS) with sodium azide. Intracellular fixation buffer was added before incubation

with primary (1:1000) and secondary antibodies (1:400) diluted in permeabilization buffer according to the manufacturer's protocol. Antibodies against flag (mouse anti-flag antibody) and HA (rabbit anti-HA antibody) were obtained from Sigma-Aldrich (St Louis, MO, USA), while antibodies against the myc epitope (goat anti-myc antibody) was obtained from Abcam (Cambridge, UK). Secondary antibodies against mouse immunoglobulin G (IgG), goat IgG and rabbit IgG were conjugated with either Alexa Fluor 488 or 594 obtained from Molecular Probes (Life Technologies). Hoechst trihydrochloride trihydrate (Life Technologies) was used for nuclear staining. The cover slips were mounted onto glass slides using Fluoroshield (Sigma-Aldrich) and prepared for microscopy as described above. Images were captured on an inverted fluorescence microscope (Olympus IX81) and on a confocal laser scanning microscope (Zeiss LSM 710).

Immunoprecipitation

A total of 5 million EPC cells were pelleted by centrifugation, resuspended in 100 μ L Ingenio Electroporation Solution (Mirus, Madison, WI, USA) and co-transfected with 8 μ g plasmid using the Amaxa T-20 program. pcDNA3.1- μ NS-N-FLAG was co-transfected with pcDNA3.1- σ NS-N-MYC, pcDNA3.1- μ 2-C-HA, pcDNA3.1- λ 1-N-HA and pcDNA3.1 S8ORF2 (negative control) separately, using three parallel preparations. The transfected cells were transferred to 75 cm² culture flasks containing 20 mL pre-equilibrated L-15 growth medium (described above). From each culture flask, 0.5 mL transfected cells were transferred to a 24-well plate intended for expression analysis by immunofluorescence microscopy. Cells were collected from the culture flasks 72 h post transfection (hpt), centrifuged at 5000 *g* for 5 min and resuspended in 1 mL Nonidet-P40 lysis buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA) containing Complete ultra mini protease inhibitor cocktail (Roche, Mannheim, Germany). The mix was incubated on ice for 30 min, and then centrifuged at 9700 *g* for 12 min at 4 °C. The supernatant was transferred to a new tube, added antibodies against the desired epitope tag or anti-S8ORF2 and incubated overnight at 4 °C with rotation. The Immunoprecipitation Kit Dynabeads Protein G (Novex, Life Technologies) was used for protein extraction and the beads prepared according to the manufacturer's protocol. The cell-lysate-antibody mixture was mixed with the protein G coated beads and incubated 2 h at 4 °C. The beads-antibody-protein complex was washed according to the manufacturer's protocol.

Western blotting

The beads-antibody-protein complex was diluted in Sample Buffer (Bio-Rad, Hercules, CA, USA) and

Reducing Agent (Bio-Rad), denatured for 5 min at 95 °C and run in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 4-12% Bis-Tris Criterion XT gel (Bio-Rad). Lysates from non-transfected EPC cells were used as a negative control, and Precision Plus Protein Western C Standards (Bio-Rad) as a molecular size marker. Following SDS-PAGE, the proteins were blotted onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) and incubated with primary antibody (anti-flag 1:1000) at 4 °C overnight. After incubation with secondary antibody (Anti-mouse IgG-HRP, GE Healthcare, Buckinghamshire, UK), the proteins were detected by chemiluminescence using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare).

Results

Prediction of secondary structure

The predicted secondary structure profiles of PRV and MRV μ NS were similar despite low sequence identity (Figure 2). The PRV μ NS sequence in this study differs by twenty-three nucleotides of which twenty are silent (not shown) to that analyzed in a previous study (GU994018) [8]. The three amino acids that differed between the two PRV μ NS sequences did not cause significant changes to the predicted secondary structures as determined by the PSIPRED program. The remaining three nucleotides all result in synonymous amino acid differences, i.e., displaying similar physiochemical properties (M/L₉₄, I/V₄₅₁ and A/V₄₉₈). For σ NS, the difference is six nucleotides and for λ 1 twenty-eight, all silent. For μ 2, the difference is fifteen nucleotides, all silent except for one synonymous substitution (R/K₁₁₃).

μ NS forms viral factory-like structures

EPC cells transfected with pcDNA3.1- μ NS-N-FLAG 48 hpt showed small, dense globular inclusions evenly distributed in the cytoplasm with some larger perinuclear inclusions 48 hpt (Figure 3A). A similar staining pattern was seen with the corresponding C-terminally flag-labelled construct (Figure 3A, insert), and in CHSE cells (not shown). EPC cells transfected with the σ NS-N-MYC, μ 2-C-HA or λ 1-N-HA constructs were also examined 48 hpt (Figure 3B–D). The σ NS-N-MYC protein was evenly distributed in the cytoplasm possibly with some minor nuclear localization (Figure 3B). A nucleocytoplasmic distribution pattern was also observed with the C-terminally myc-labelled σ NS (Figure 3B, insert). Both the μ 2-C-HA and λ 1-N-HA proteins were evenly distributed in the cytoplasm (Figure 3C and D), with the former showing minor staining in the nucleus of some cells (not shown). Non-transfected cells did not show any staining (not shown).

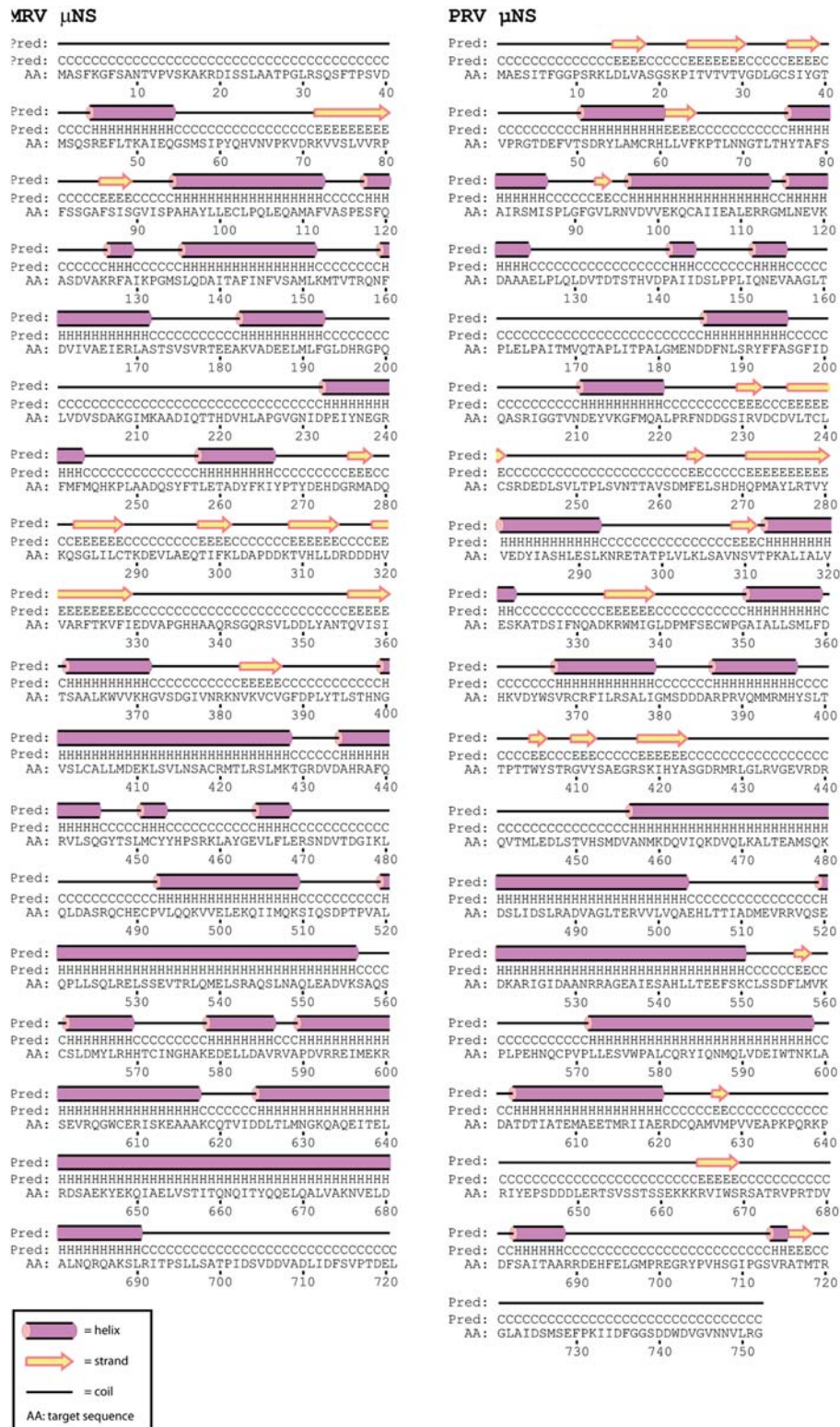


Figure 2 Secondary structure predictions. Secondary structure predictions of the μNS proteins from PRV and MRV (PSIPRED). Accession numbers for the MRV and PRV proteins are NC004281 and KR337478, respectively.

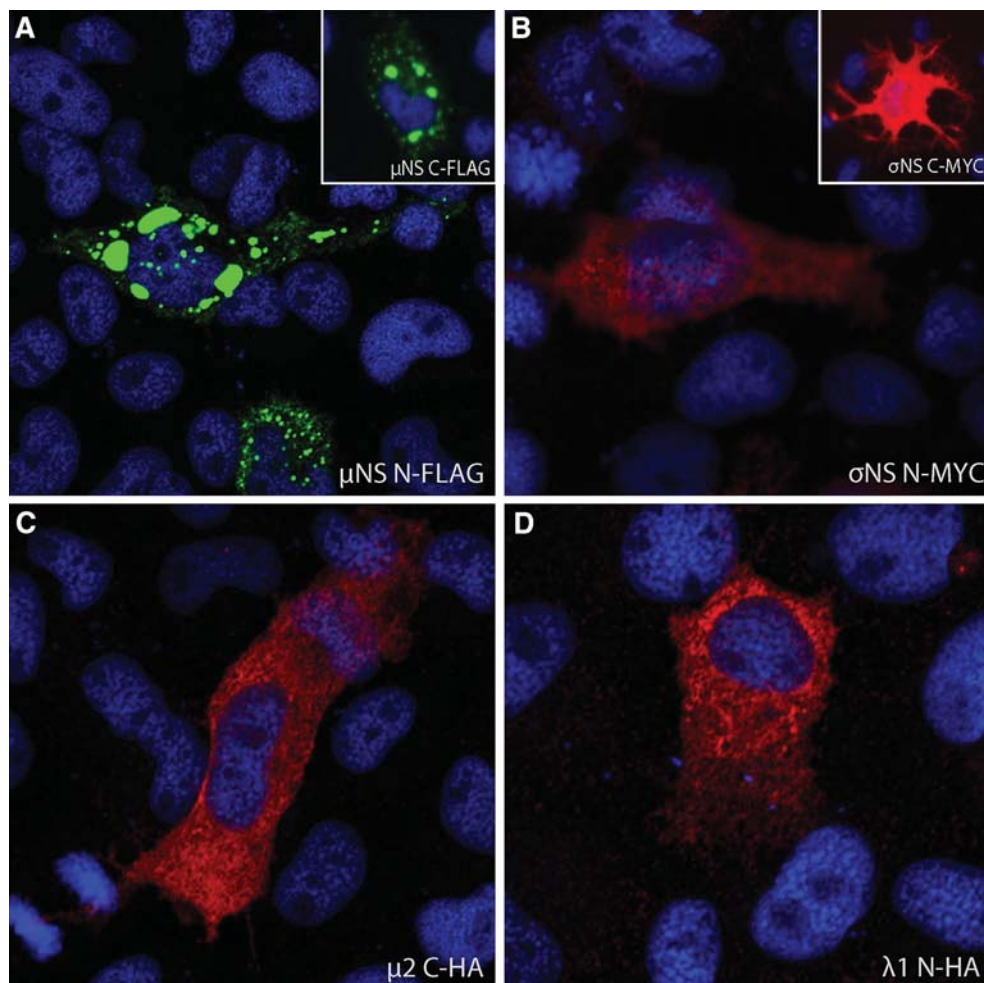


Figure 3 Subcellular localization of PRV proteins. EPC cells transfected with four different PRV plasmid constructs (μ NS, σ NS, λ 1, μ 2) processed for fluorescence microscopy 48 hpt. **A** EPC cells expressing μ NS N-FLAG. Boxed region in top left corner shows EPC cells expressing μ NS-C-FLAG. **B** EPC cells expressing σ NS N-MYC. Boxed region shows σ NS-C-MYC. **C** EPC cells expressing μ 2-C-HA. **D** EPC cells expressing λ 1-N-HA.

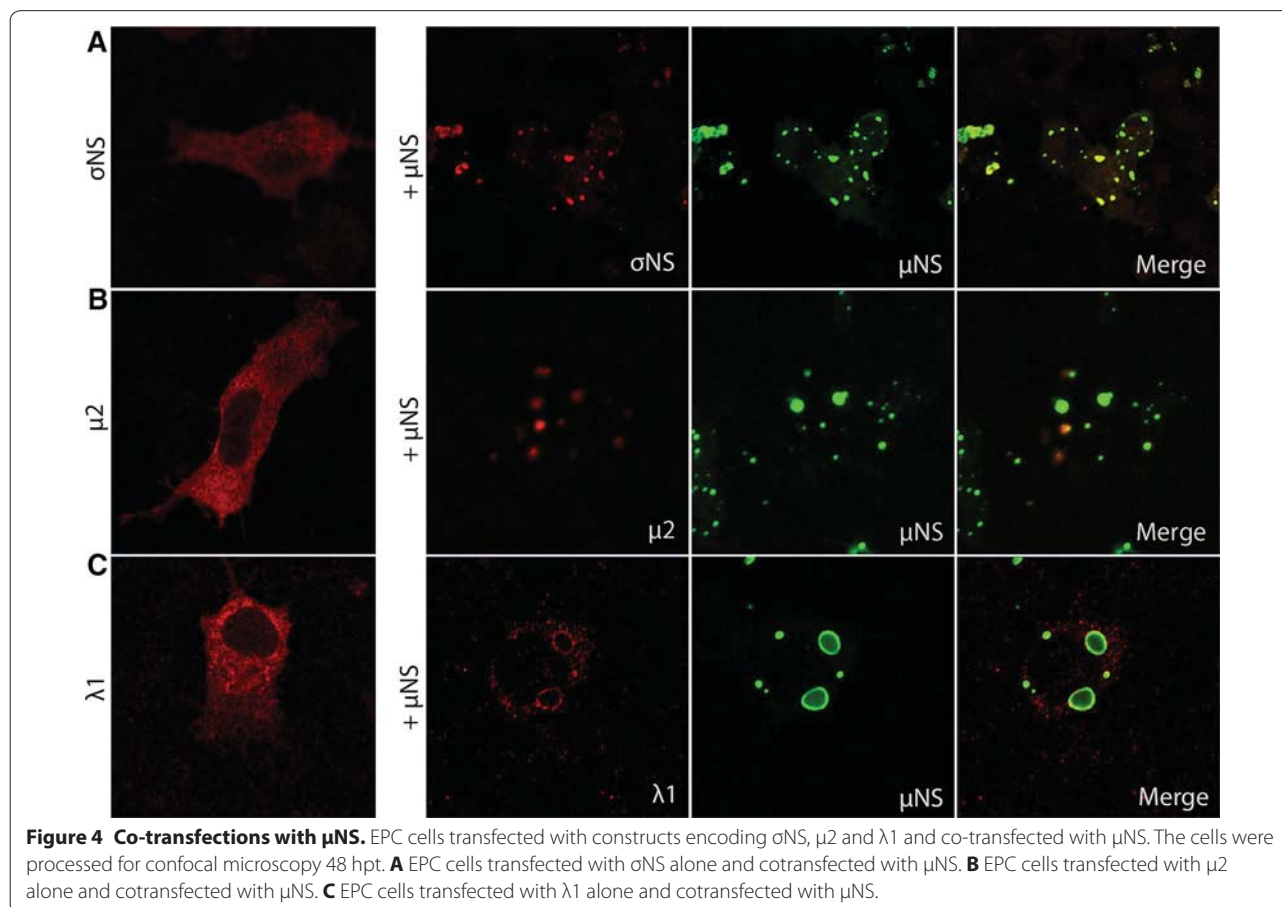
σ NS, λ 1 and μ 2 are recruited to viral factory-like structures

Viral proteins interacting with μ NS were identified by co-transfecting EPC cells with pcDNA3.1- μ NS-N-FLAG and separately with each of the σ NS-N-MYC, μ 2-C-HA or λ 1-N-HA constructs. The μ NS protein retained its globular distribution pattern in the presence of the other PRV proteins 48 hpt (Figure 4). In contrast, the staining pattern for σ NS, μ 2 and λ 1 proteins changed from an evenly cytoplasmic distribution to globular inclusions co-localizing wholly or partially with the μ NS protein (Figure 4A–C). Co-localization with μ NS was most pronounced for σ NS, and σ NS was no longer found in the nucleus (Figure 4A). For μ 2, the change in distribution was not as pronounced as for σ NS and λ 1, but in some cells μ 2 formed small punctuated structures partially overlapping with the μ NS

globular inclusions (Figure 4B). Co-expression of σ NS-N-MYC with either μ 2-C-HA or λ 1-N-HA, i.e. in the absence of μ NS, did not alter staining patterns, and the viral factory-like structures were not formed (not shown).

σ NS and μ 2 interact with μ NS

Immunoprecipitation and western blotting were performed to confirm interactions between PRV μ NS and each of σ NS, λ 1 and μ 2 (Figure 5). EPC cells were co-transfected with μ NS-N-FLAG and separately with the σ NS-N-MYC, λ 1-N-HA and μ 2-C-HA constructs. The results confirmed that μ NS interacts with σ NS and μ 2. Interaction with λ 1 on the other hand (Figure 5), or to the negative control ISAV-S8ORF2 protein, was not observed (not shown).



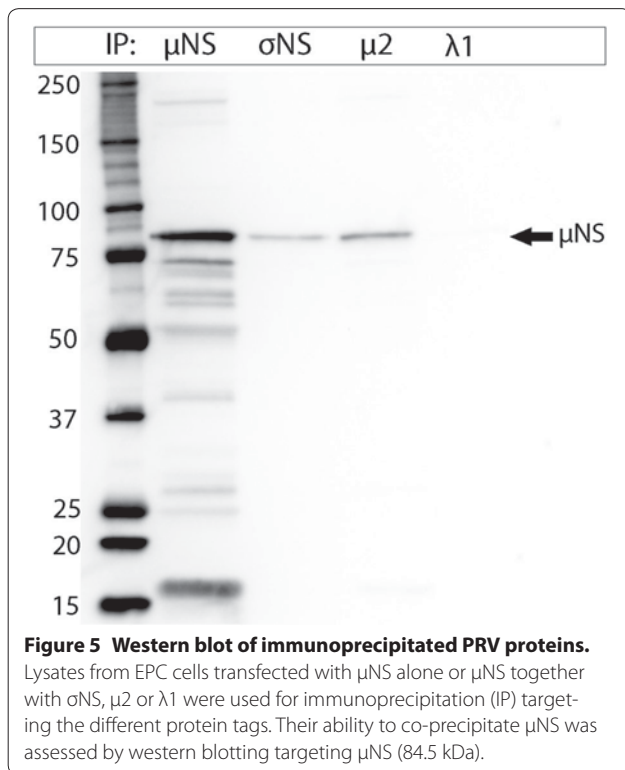
Truncated μ NS proteins

EPC cells were transfected with plasmid constructs encoding the truncated μ NS variants μ NS- Δ 743-752, μ NS- Δ 736-752, μ NS- Δ 1-401 and μ NS- Δ 402-752 (Figure 1). Small, factory-like globular inclusions were formed by μ NS Δ 743-752 and μ NS Δ 736-752 (Figure 6A and B). Individual co-expression of these μ NS truncated variants with σ NS-N-MYC recruited the latter protein to the factory-like inclusions, similar to that observed with full-length μ NS (Figures 4A, 6A and B). The μ NS Δ 1-401 protein formed small dense irregular or granular structures in the cytoplasm with reminiscences to the globular structures formed by the full-length protein (Figure 6C). The μ NS Δ 1-401 truncated version did also recruit and change the distribution pattern of σ NS (Figures 3B and 6C). In contrast, μ NS Δ 402-752 was evenly distributed in the cytoplasm, and did not form viral factory-like structures. When μ NS Δ 402-752 was expressed together with σ NS, both proteins were evenly dispersed throughout the cytoplasm (Figure 6D).

Discussion

The reoviral factories are the sites for virus replication and particle assembly [19]. The MRV μ NS is the

scaffolding protein organizing the viral factories including gathering of core proteins, while the σ NS protein facilitates construction of core particles and subsequent particle assembly [20, 24, 29, 34]. Viral factory-like structures have been observed in PRV infected Atlantic salmon erythrocytes in both in vivo and ex vivo experiments [22, 23]. In this study we demonstrated that PRV μ NS alone forms dense globular, viral factory-like cytoplasmic inclusions. The globular, cytoplasmic distribution of μ NS was not seen for the non-structural σ NS or the structural μ 2 and λ 1 PRV proteins. However, these proteins changed their distribution pattern and co-localized with μ NS in the dense globular structures when they were co-transfected with μ NS. Co-transfection of σ NS with μ 2 or λ 1 did not cause changes in distribution pattern. Expression of the N-terminal 401 amino acids did not form viral factory-like structures, mapping this feature to the remaining C-terminal 351 amino acids. Immunoprecipitation and subsequent Western blot analysis confirmed the association between μ NS- σ NS and μ NS- μ 2. Our findings strongly suggests that μ NS is the prime organizer of viral factories for PRV.



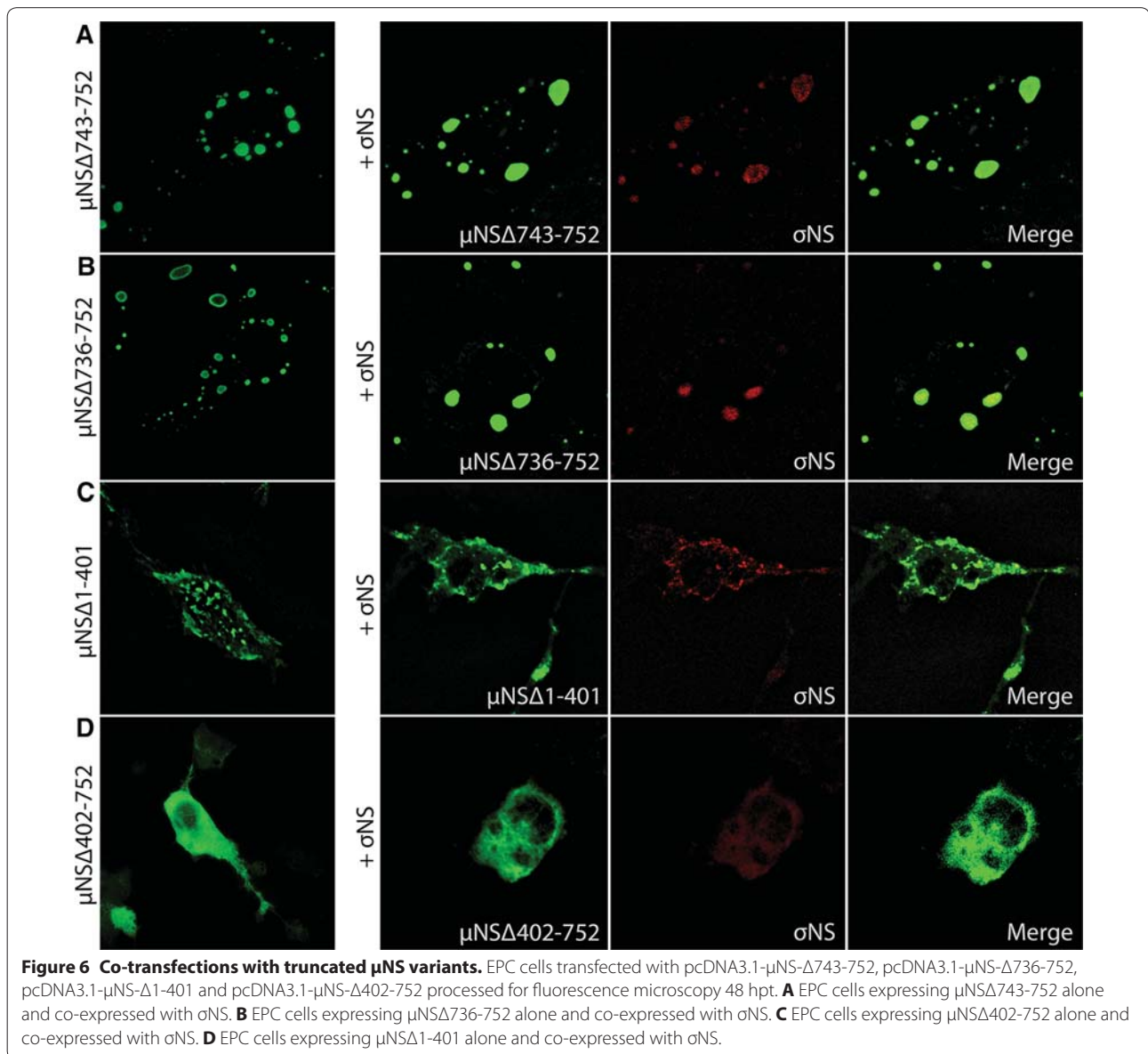
MRV strains exhibit differences in viral inclusion morphology. Reovirus type 1 Lang (T1L) forms filamentous inclusions, whereas type 3 Dearing (T3D) forms punctate or globular inclusions [20, 35]. These morphologic differences are determined by the ability of the virus to interact with the microtubule system, a feature mapped to MRV μ 2 [35]. In the filamentous factories, μ 2 co-localize with and stabilize microtubules when expressed in cells in the absence of other viral proteins [20, 35]. PRV inclusions appear similar to the globular inclusion type, closely resembling the μ NS-containing globular viral factories in reovirus T3D infected cells [35]. We cannot exclude that there are strains of PRV that forms filamentous inclusions. There might be several not yet recognized PRV-like viruses that infect other salmonid fish species. It has been proposed that the larger surface area of filamentous inclusions allow for more efficient viral replication through better access to small-molecule substrates or newly synthesized proteins from the surrounding cytosol [35]. Immunofluorescence and confocal microscopy have been used to identify globular and filamentous inclusions after transfection with expression plasmids encoding proteins from MRV and ARV [27, 36, 37].

Viral factories commonly form early in reovirus infection as small punctate structures throughout the cytoplasm that increase in size and become more perinuclear during infection [20]. The factories recruit viral proteins,

which allow the efficient assembly of virus core particles [34, 38]. We observed that PRV μ NS guided the σ NS, μ 2 and λ 1 proteins to the viral factories. Our rationale for choosing σ NS, μ 2 and λ 1 as co-transfectants was that these are examples of non-structural (σ NS) and structural (μ 2 and λ 1) proteins in the core particle. MRV μ NS and σ NS are found in the first detectable viral protein-RNA complexes in MRV infected cells and form cytoplasmic inclusions similar to the viral factory-like structures formed in the absence of viral infection [36]. Analysis of MRV μ NS transfected cells revealed that at 6 hpt, μ NS inclusions were uniformly small and spread throughout the cytoplasm, whereas at 18 hpt and 36 hpt, larger perinuclear inclusions were present along with smaller inclusions [20]. In addition to its association with σ NS, MRV μ NS has been shown to interact with each of the five structural proteins that make up the core particle (λ 1, λ 2, λ 3, σ 2 and μ 2) [24, 34]. Although it generally occurs within 18 hpt, strong co-localization between MRV μ NS and the core surface proteins have been observed as soon as 6 h post infection [34]. Since PRV replicates at lower temperatures than MRV, the process of assembling core proteins to viral factories occurs at a slower rate. Studies on the ARV have identified a similar role of μ NS in forming viral factories [27].

The nature of the globular inclusions and their interactions with other PRV proteins might differ in erythrocytes and established cell lines. However, neither cell line nor C- or N-terminal epitope tagging influenced the formation of dense globular structures by the PRV μ NS. Transfection of salmon erythrocytes was not successful (data not shown). Still, globular-type inclusions are common in naturally PRV infected erythrocytes. This indicates that the formation of globular inclusion structures is an intrinsic property of μ NS.

The ability of μ NS to redirect the subcellular localizations of other PRV proteins can be mediated through protein-protein interactions. This was observed for σ NS and μ 2 following immunoprecipitation and western blotting. Many cellular proteins are only functional when localized to specific cellular compartments, and translocation to the appropriate sites can serve to regulate protein function [36]. Reovirus proteins involved in replication are only active within functional centers characterized by a particular location and protein composition [36]. We could not demonstrate protein-protein interaction between μ NS and λ 1, although confocal imaging clearly proved redistribution of λ 1 when the protein was co-expressed with μ NS. Interaction(s) between μ NS and λ 1 is therefore likely but perhaps through the involvement of a third cellular protein. Alternatively, the binding affinities between the two proteins are below the threshold detectable by the conditions used in the



immunoprecipitation- and western blot assays. Further investigations are needed to study the mechanisms involved in λ 1 redistribution when co-expressed with μ NS. Since μ NS expressed alone forms viral factory-like inclusions, and is responsible for the redistribution of other PRV proteins, it is likely one of the first proteins involved in virus factory formation and thereby essential in the early steps of viral replication.

Staining of σ NS, and to some extent μ 2, was observed in the nucleus of transfected cells. The size of the σ NS protein, predicted to be 39.1 kDa, may allow passive diffusion through the nuclear pores, whereas the 86 kDa μ 2 protein exceeds the 40 kDa limit for passive

diffusion [39]. MRV σ NS and μ 2 are both shown to be distributed in the nucleus and the cytoplasm of transfected and infected cells. The ability of MRV σ NS to locate in the nucleus of infected cells has been linked to its nucleic acid binding capability, while the presence of MRV μ 2 in the nucleus of transfected cells is explained by predicted nuclear import and export signals [20, 24, 40–42]. There are no predicted classical nuclear localization signals (NLSs) in PRV σ NS [8] or PRV μ 2 (present study, using PSORTII and NLS mapper). The presence of nuclear export signals (NES) have though been predicted for both proteins. Neither σ NS nor μ 2 was found in the nucleus after co-transfection with μ NS. As μ NS does not

localize to the nucleus, an explanation might be that μ NS sequesters σ NS and μ 2 within the cytoplasmic inclusions, thus reducing the amount of free σ NS and μ 2 to enter the nucleus. This has also been proposed for MRV σ NS and μ 2 [20, 40]. Further studies are needed to excavate the functional roles of the observed nuclear localization of PRV σ NS and μ 2.

The C-terminal part of MRV μ NS contains four distinct regions comprising 250 amino acids that are sufficient to form viral factories [29]. These regions include two predicted coiled-coil domains, a linker region between the coiled coils containing a putative zinc hook, and a short C-terminal tail [24]. PRV μ NS may contain a coiled-coil motif in its C-terminal region [8]. A deletion of the eight C-terminal amino acids of MRV μ NS results in diffusely distributed protein throughout the cytoplasm and the nucleus, suggesting that these amino acids are necessary for inclusion formation [29]. PRV μ NS also contains a high α -helical content in its C-terminal region although the sequence identity to the homologous MRV protein is low [8]. In fact, the predicted secondary structure profiles of MRV and PRV μ NS show significant similarities, highlighting the importance of conserving structural features over primary sequence for the function of homologous proteins across evolutionary lines. Still, the two C-terminally truncated forms of μ NS containing deletions of 10 and 17 amino acids, respectively, formed viral factory-like structures when expressed in EPC cells, indicating that factory formation is not dependent on these amino acids. Deletion of the 401 N-terminal amino acids seemed to have some influence on the viral factory formation, but the protein still accumulated in granular structures and retained its ability to recruit σ NS. Deletions of the 351 C-terminal amino acids, on the other hand, resulted in diffusely distributed protein and absence of globular inclusions. This indicates that the C-terminal region of μ NS is essential for factory formation. The N-terminal region of PRV μ NS displays a somewhat higher level of secondary structure conservation when compared to MRV. In MRV, this region of μ NS is crucial for interactions with σ NS, μ 2, λ 1 and λ 2 [34, 38].

In conclusion, our results strongly suggest that PRV μ NS protein is essential for factory formation and assembly of viral proteins, similar to that of μ NS of other orthoreoviruses. Further studies on both the structural and functional properties of PRV proteins can provide important information relating to disease development following PRV infections.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HH constructed the expression plasmids, performed the transfections, the immunofluorescence microscopy, the immunoprecipitation and western

blot analyses and drafted the manuscript. IN participated in the construction of expression plasmids and in the immunoprecipitation and western blot analyses. TM carried out the computer analyses and participated in the construction of truncated proteins. ØW participated in the construction of expression plasmids and in the design of the study. MD and ER conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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References

- Palacios G, Løvoll M, Tengs T, Hornig M, Hutchison S, Hui J, Kongtorp RT, Savji N, Bussetti AV, Solovyov A, Kristoffersen AB, Celeone C, Street C, Trifonov V, Hirschberg DL, Rabadan R, Egholm M, Rimstad E, Lipkin WI (2010) Heart and skeletal muscle inflammation of farmed salmon is associated with infection with a novel reovirus. *PLoS One* 5:e11487
- Finstad ØW, Falk K, Løvoll M, Evensen Ø, Rimstad E (2012) Immunohistochemical detection of piscine reovirus (PRV) in hearts of Atlantic salmon coincide with the course of heart and skeletal muscle inflammation (HSMI). *Vet Res* 43:27
- Kongtorp RT, Halse M, Taksdal T, Falk K (2006) Longitudinal study of a natural outbreak of heart and skeletal muscle inflammation in Atlantic salmon, *Salmo salar* L. *J Fish Dis* 29:233–244
- Løvoll M, Alarcon M, Bang Jensen B, Taksdal T, Kristoffersen AB, Tengs T (2012) Quantification of piscine reovirus (PRV) at different stages of Atlantic salmon *Salmo salar* production. *Dis Aquat Organ* 99:7–12
- Rimstad E (2011) Examples of emerging virus diseases in salmonid aquaculture. *Aquac Res* 42:86–89
- Garseth AH, Fritsvold C, Opheim M, Skjerve E, Biering E (2013) Piscine reovirus (PRV) in wild Atlantic salmon, *Salmo salar* L., and sea-trout, *Salmo trutta* L., in Norway. *J Fish Dis* 36:483–493
- Nibert ML, Duncan R (2013) Bioinformatics of recent aqua- and orthoreovirus isolates from fish: evolutionary gain or loss of FAST and fiber proteins and taxonomic implications. *PLoS One* 8:e68607
- Markussen T, Dahle MK, Tengs T, Løvoll M, Finstad ØW, Wiik-Nielsen CR, Grove S, Lauksund S, Robertsen B, Rimstad E (2013) Sequence analysis of the genome of piscine orthoreovirus (PRV) associated with heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (*Salmo salar*). *PLoS One* 8:e70075
- Kibenge MJ, Iwamoto T, Wang Y, Morton A, Godoy MG, Kibenge FS (2013) Whole-genome analysis of piscine reovirus (PRV) shows PRV represents a new genus in family reoviridae and its genome segment S1 sequences group it into two separate sub-genotypes. *Virology* 453:220–230
- Wessel Ø, Nyman IB, Markussen T, Dahle MK, Rimstad E (2015) Piscine orthoreovirus (PRV) σ 3 protein binds dsRNA. *Virus Res* 198:22–29
- Key T, Read J, Nibert ML, Duncan R (2013) Piscine reovirus encodes a cytotoxic, non-fusogenic, integral membrane protein and previously unrecognized virion outer-capsid proteins. *J Gen Virol* 94:1039–1050
- Guglielmi KM, Johnson EM, Stehle T, Dermody TS (2006) Attachment and cell entry of mammalian orthoreovirus. *Curr Top Microbiol* 309:1–38
- Novoa RR, Calderita G, Arranz R, Fontana J, Granzow H, Risco C (2005) Virus factories: associations of cell organelles for viral replication and morphogenesis. *Biol Cell* 97:147–172

14. Becker MM, Peters TR, Dermody TS (2003) Reovirus σ NS and μ NS proteins form cytoplasmic inclusion structures in the absence of viral infection. *J Virol* 77:5948–5963
15. Schiff LA, Nibert ML, Tyler KL (2007) Orthoreoviruses and their replication. In: Knipe DM, Howley PM, Fields BN (eds) *Fields virology*, vol 2, 5th edn. Wolters Kluwer/Lippincott Williams & Wilkins, Philadelphia
16. Paul D, Bartenschlager R (2013) Architecture and biogenesis of plus-strand RNA virus replication factories. *World J Virol* 2:32–48
17. Netherton C, Moffat K, Brooks E, Wileman T (2007) A guide to viral inclusions, membrane rearrangements, factories, and viroplasm produced during virus replication. *Adv Virus Res* 70:101–182
18. de Castro IF, Volonte L, Risco C (2013) Virus factories: biogenesis and structural design. *Cell Microbiol* 15:24–34
19. Fernandez de Castro I, Zamora PF, Ooms L, Fernandez JJ, Lai CM, Mainou BA, Dermody TS, Risco C (2014) Reovirus forms neo-organelles for progeny particle assembly within reorganized cell membranes. *MBio* 5:e00931–e01013
20. Broering TJ, Parker JS, Joyce PL, Kim J, Nibert ML (2002) Mammalian reovirus nonstructural protein microNS forms large inclusions and colocalizes with reovirus microtubule-associated protein micro2 in transfected cells. *J Virol* 76:8285–8297
21. Schmid M, Speiseder T, Dobner T, Gonzalez RA (2014) DNA virus replication compartments. *J Virol* 88:1404–1420
22. Finstad ØW, Dahle MK, Lindholm TH, Nyman IB, Løvoll M, Wallace C, Olsen CM, Storset AK, Rimstad E (2014) Piscine orthoreovirus (PRV) infects Atlantic salmon erythrocytes. *Vet Res* 45:35
23. Wessel Ø, Olsen CM, Rimstad E, Dahle MK (2015) Piscine orthoreovirus (PRV) replicates in Atlantic salmon (*Salmo Salar* L.) erythrocytes ex vivo. *Vet Res* 46:26
24. Miller CL, Arnold MM, Broering TJ, Hastings CE, Nibert ML (2010) Localization of mammalian orthoreovirus proteins to cytoplasmic factory-like structures via nonoverlapping regions of microNS. *J Virol* 84:867–882
25. Ivanovic T, Boulant S, Ehrlich M, Demidenko AA, Arnold MM, Kirchhausen T, Nibert ML (2011) Recruitment of cellular clathrin to viral factories and disruption of clathrin-dependent trafficking. *Traffic* 12:1179–1195
26. McCutcheon AM, Broering TJ, Nibert ML (1999) Mammalian reovirus M3 gene sequences and conservation of coiled-coil motifs near the carboxyl terminus of the microNS protein. *Virology* 264:16–24
27. Touris-Otero F, Cortez-San Martin M, Martinez-Costas J, Benavente J (2004) Avian reovirus morphogenesis occurs within viral factories and begins with the selective recruitment of sigmaNS and lambdaA to microNS inclusions. *J Mol Biol* 341:361–374
28. Brandariz-Nunez A, Menaya-Vargas R, Benavente J, Martinez-Costas J (2010) Avian reovirus microNS protein forms homo-oligomeric inclusions in a microtubule-independent fashion, which involves specific regions of its C-terminal domain. *J Virol* 84:4289–4301
29. Broering TJ, Arnold MM, Miller CL, Hurt JA, Joyce PL, Nibert ML (2005) Carboxyl-proximal regions of reovirus nonstructural protein muNS necessary and sufficient for forming factory-like inclusions. *J Virol* 79:6194–6206
30. Busch LK, Rodriguez-Grille J, Casal JI, Martinez-Costas J, Benavente J (2011) Avian and mammalian reoviruses use different molecular mechanisms to synthesize their microNS isoforms. *J Gen Virol* 92:2566–2574
31. Yachdav G, Kloppmann E, Kajan L, Hecht M, Goldberg T, Hamp T, Honigschmid P, Schafferhans A, Roos M, Bernhofer M, Richter L, Ashkenazy H, Punta M, Schlessinger A, Bromberg Y, Schneider R, Vriend G, Sander C, Ben-Tal N, Rost B (2014) PredictProtein—an open resource for online prediction of protein structural and functional features. *Nucl Acids Res* 42:W337–W343
32. Terpe K (2003) Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol* 60:523–533
33. Garcia-Rosado E, Markussen T, Kileng O, Baekkevold ES, Robertsen B, Mjaaland S, Rimstad E (2008) Molecular and functional characterization of two infectious salmon anaemia virus (ISAV) proteins with type I interferon antagonizing activity. *Virus Res* 133:228–238
34. Broering TJ, Kim J, Miller CL, Piggott CD, Dinoso JB, Nibert ML, Parker JS (2004) Reovirus nonstructural protein muNS recruits viral core surface proteins and entering core particles to factory-like inclusions. *J Virol* 78:1882–1892
35. Parker JS, Broering TJ, Kim J, Higgins DE, Nibert ML (2002) Reovirus core protein mu2 determines the filamentous morphology of viral inclusion bodies by interacting with and stabilizing microtubules. *J Virol* 76:4483–4496
36. Becker MM, Peters TR, Dermody TS (2003) Reovirus sigmaNS and muNS proteins form cytoplasmic inclusion structures in the absence of viral infection. *J Virol* 77:5948–5963
37. Becker MM, Goral MI, Hazelton PR, Baer GS, Rodgers SE, Brown EG, Coombs KM, Dermody TS (2001) Reovirus sigmaNS protein is required for nucleation of viral assembly complexes and formation of viral inclusions. *J Virol* 75:1459–1475
38. Carroll K, Hastings C, Miller CL (2014) Amino acids 78 and 79 of mammalian orthoreovirus protein microNS are necessary for stress granule localization, core protein lambda2 interaction, and de novo virus replication. *Virology* 448:133–145
39. Mohr D, Frey S, Fischer T, Guttler T, Gorlich D (2009) Characterisation of the passive permeability barrier of nuclear pore complexes. *EMBO J* 28:2541–2553
40. Gillian AL, Nibert ML (1998) Amino terminus of reovirus nonstructural protein sigmaNS is important for ssRNA binding and nucleoprotein complex formation. *Virology* 240:1–11
41. Kobayashi T, Ooms LS, Chappell JD, Dermody TS (2009) Identification of functional domains in reovirus replication proteins muNS and mu2. *J Virol* 83:2892–2906
42. Ooms LS, Kobayashi T, Dermody TS, Chappell JD (2010) A post-entry step in the mammalian orthoreovirus replication cycle is a determinant of cell tropism. *J Biol Chem* 285:41604–41613

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III

Article

Viral Protein Kinetics of Piscine Orthoreovirus Infection in Atlantic Salmon Blood Cells

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Abstract: *Piscine orthoreovirus* (PRV) is ubiquitous in farmed Atlantic salmon (*Salmo salar*) and the cause of heart and skeletal muscle inflammation. Erythrocytes are important target cells for PRV. We have investigated the kinetics of PRV infection in salmon blood cells. The findings indicate that PRV causes an acute infection of blood cells lasting 1–2 weeks, before it subsides into persistence. A high production of viral proteins occurred initially in the acute phase which significantly correlated with antiviral gene transcription. Globular viral factories organized by the non-structural protein μ NS were also observed initially, but were not evident at later stages. Interactions between μ NS and the PRV structural proteins λ 1, μ 1, σ 1 and σ 3 were demonstrated. Different size variants of μ NS and the outer capsid protein μ 1 appeared at specific time points during infection. Maximal viral protein load was observed five weeks post cohabitant challenge and was undetectable from seven weeks post challenge. In contrast, viral RNA at a high level could be detected throughout the eight-week trial. A proteolytic cleavage fragment of the μ 1 protein was the only viral protein detectable after seven weeks post challenge, indicating that this μ 1 fragment may be involved in the mechanisms of persistent infection.

Keywords: *Piscine orthoreovirus*; PRV; non-structural protein; μ NS; μ 1; expression kinetics; proteolytic cleavage; pathogenesis; blood cells; Atlantic Salmon

1. Introduction

Piscine orthoreovirus (PRV) belongs to the genus *Orthoreovirus* in the family *Reoviridae* [1,2]. The orthoreoviruses are ubiquitous in various animal species, but only found to be of pathogenic significance in poultry and recently in fish [3–7]. PRV is abundant in farmed Atlantic salmon (*Salmo salar*), detected both in apparently healthy and diseased fish [8–11]. The infection causes heart and skeletal muscle inflammation (HSMI) and is associated with melanised foci in white muscle in Atlantic salmon [1,7,12]. HSMI is a prevalent disease and melanised foci is a quality problem; both conditions are of major economic importance to salmon aquaculture. The pathogenesis of HSMI is not completely elucidated. Outbreaks of the disease are primarily observed in the seawater phase and last for several weeks in the population [13], after which the PRV infection becomes persistent [9,11,14]. In experimental cohabitant infection trials, disease onset occurs after 8–10 weeks [15].

The study of molecular mechanisms linked to PRV infection has been limited by the lack of susceptible cell lines. Studies of the viral infection have therefore been performed *in vivo* or by infecting erythrocytes *ex vivo* [16]. Piscine erythrocytes are nucleated and contain the transcriptional and translational machinery necessary for expression of mRNA and proteins [17]. Erythrocytes are important target cells for PRV and the infection activates an innate antiviral immune response typical for RNA viruses in these cells [18]. During the peak phase of infection, more than 50% of all erythrocytes may be infected [19]. Interestingly, severe anemia has not been reported from HSMI outbreaks in the seawater phase, indicating low or no virus-induced lysis of infected erythrocytes [20]. Recently, a variant of PRV was demonstrated to be the etiologic agent of erythrocytic inclusion body syndrome (EIBS), a condition associated with anemia and mass mortality in juvenile Coho salmon (*Onchorhynchus kisutchi*). The level of anemia in EIBS affected fish corresponded with the level of viral replication in blood [7]. In addition, infection of rainbow trout in fresh water by yet another PRV variant is also associated with anemia and an HSMI-like disease [5].

The *Orthoreovirus* genome consists of ten double-stranded RNA (dsRNA) segments enclosed in a double protein capsid. The genomic segments are classified according to size with three large (L), three medium (M) and four small (S) segments encoding the λ , μ and σ class proteins, respectively [3,21]. In mammalian orthoreovirus (MRV), the species type of genus *Orthoreovirus*, the viral transcription machinery is located in the inner core and consists of $\lambda 1$, $\lambda 2$, $\lambda 3$, $\mu 2$ and $\sigma 2$ [22]. The outer capsid proteins $\mu 1$, $\sigma 1$ and $\sigma 3$ are involved in cell attachment and membrane penetration during the initial stages of infection [23–25]. The two non-structural proteins μNS and σNS participate in the formation of viral factories where viral genome replication and particle assembly occur [21,26,27]. Although some important amino acid motifs are conserved between MRV and PRV, sequence identities between homologous proteins are generally low [2]. MRV enters the cell by receptor-mediated endocytosis. The outer capsid is largely removed and $\mu 1$ is cleaved at two positions that generate, in addition to the full-length protein, five different fragments [24,28]. The N-terminal autolytic cleavage site, which produces $\mu 1N$ and $\mu 1C$, seems conserved across orthoreoviruses, including PRV [2,29,30]. Further cleavage of $\mu 1C$, mediated by exogenous proteases, generate fragments δ and ϕ [24].

Structures resembling viral factories have also been observed in PRV-infected erythrocytes, and recombinant expression of the protein in fish cell lines indicate that PRV μNS has an analogous role in factory formation [16,19,31]. The majority of virus-encoded proteins localize completely or partially within these viral factories [2,3,21]. The viral factories in PRV-infected cells resemble the globular structures observed for the MRV type 3 Dearing (T3D) strain, in contrast to the filamentous-like viral factories generated by MRV Type 1 Lang (T1L) [19]. The latter is considered the most common morphology type of orthoreoviral factories [21,32]. Gene segment M3 in MRV and avian orthoreovirus (ARV) are reported to produce two isoforms of the factory forming μNS protein in infected cells [33–35]. The second isoform is produced by different mechanisms in the two viruses; in MRV, μNSC is expressed by a second in-frame AUG (Met₄₁) while in ARV, post-translational cleavage in the N-terminal region releases μNSN [33,35,36]. In ARV, only full-length μNS interacts with σNS in infected cells, suggesting that the two isoforms play different roles during ARV infection [34].

Considering the emerging occurrence of HSMI, PRV exhibits a considerable risk for the aquaculture industry and proper disease control is highly desired. To understand the association between PRV infection and disease outcome, and also to limit further disease outbreaks, more information regarding PRV protein kinetics is essential. In the present study, the kinetics of viral RNA, viral protein and antiviral immune response in blood cells from experimentally PRV-infected Atlantic salmon were investigated. We hypothesized that PRV causes an acute infection in blood cells correlating with innate antiviral gene expression, before the infection subsides to a low persistent level.

2. Materials and Methods

2.1. Construction and Expression of Recombinant Piscine orthoreovirus (PRV) μ NS

Following the supplier's protocol, the BaculoDirect™ Baculovirus Expression System (Invitrogen, Carlsbad, CA, USA) was used to generate recombinant μ NS. The μ NS open reading frame (ORF) (acc. no. KR337478) was obtained by polymerase chain reaction (PCR; primers listed in Table S1) of the plasmid construct pcDNA3.1 μ NS N-FLAG [31] and cloned into the pENTR™ TOPO® vector (Invitrogen). The pENTR μ NS construct was used in a recombination reaction to generate the recombinant baculovirus DNA. Sanger sequencing (GATC Biotech AG, Konstanz, Germany) confirmed the sequence of the construct. *Spodoptera frugiperda* (Sf9) insect cells (BD Bioscience, Erembodegem, Belgium) cultured in Grace Insect Medium (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS, Life Technologies, Paisley, Scotland, UK), 100 U/mL Penicillin, 100 μ g/mL Streptomycin and 0.25 μ g/mL Fungizone (Life Technologies), were transfected with recombinant baculovirus DNA. Passage 1 (P1) viral stock was harvested 11 days post transfection and used to produce high titer viral stocks according to the supplier's protocol. The BacPAK quantitative PCR (qPCR) Titration kit (Clontech, Mountain View, CA, USA) was used to determine the viral titer. Finally, Sf9 insect cells were infected with Passage 2 (P2) or higher passage of recombinant baculovirus stock ($>1 \times 10^8$ copies/mL) and incubated at 27 °C for 96 h for expression of the recombinant μ NS protein containing a C-terminal 6xHis-tag.

2.2. Construction and Expression of Recombinant PRV λ 1

The ORF of PRV structural protein λ 1 (acc. no. KR337475) encoded by gene segment L3 was amplified (primers listed in Table S1) using cDNA originating from a HSMI outbreak [31] as template. The PCR product was cloned into pET100/D-TOPO (Invitrogen) and the sequence verified by Sanger sequencing (GATC Biotech AG). The pET100- λ 1 plasmid was transfected into *E. coli* (BL21 DE3 strain, Invitrogen) and expressed with a N-terminal 6xHis-tag, following the manufacturer's instructions. Protein expression was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Protein Purification

The Sf9 insect cells and the *E. coli* cells expressing recombinant PRV μ NS and λ 1 proteins, respectively, were pelleted by centrifugation at $5000 \times g$ for 10 min, then dissolved and washed in phosphate-buffered solution (PBS). Purification of recombinant proteins was carried out using ProBond Purification System (Life Technologies) following the manufacturer's instructions. The recombinant μ NS protein was eluted with an elution buffer containing 8 M Urea, 20 mM $\text{Na}_2\text{H}_2\text{PO}_4$ (pH 4.0), and 500 mM NaCl. The purity of the recombinant protein was monitored by SDS-PAGE using a 4%–12% Bis-Tris Criterion XT gel (Bio-Rad, Hercules, CA, USA). To purify λ 1, the Ni-NTA agarose was run on a SDS-PAGE where a band matching the size of λ 1 was excised. The gel sample containing λ 1 protein was solubilized in 250 mM Tris-HCl with 0.1% SDS, pH 6.8, sonicated 3×5 s and incubated at 4 °C with shaking overnight. The sample was centrifuged at $10,000 \times g$ for 10 min and the supernatant was dialyzed using the Slide-A-Lyser® Dialysis cassette with 20,000 molecular weight cut-off (MWCO) and 0.5–3.0 mL capacity (Thermo Scientific, Waltham, MA, USA) following the manufacturer's protocol. SDS-PAGE confirmed the purity of the recombinant λ 1 protein. Protein concentrations for both μ NS and λ 1 were determined using the DC Protein Assay Reagent Package (Bio-Rad), with bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) as protein standard.

2.4. Immunization of Rabbits

The purified recombinant proteins were used for immunization of rabbits and generation of antisera named anti- μ NS #R320684 and anti- λ 1 #K273. In the first injection, Freund's complete adjuvant was added, thereafter the rabbits were boosted three times with Freund's incomplete adjuvant

weekly. The amount of μ NS and λ 1 antigen used per immunization was in the range of 45–500 μ g. The rabbit sera produced were tested by Western blotting (WB) and fluorescent microscopy after transfection of epithelioma papulosum cyprini (EPC; ATCC CRL-2872) cells with pcDNA3.1 μ NS N-FLAG [31] or pcDNA3.1 λ 1 N-HA [31] (see description below). Antisera controls were collected prior to immunization. WB and immunofluorescent microscopy confirmed that the rabbit μ NS and λ 1 antisera recognized the μ NS and λ 1 proteins in transfected EPC cells (Figure S1). No staining was detected using the pre-immunization sera (data not shown).

2.5. Specificity of Antisera

EPC cells were cultivated in Leibovitz-15 medium (L15; Life Technologies) supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 0.04 mM mercaptoethanol and 0.05 mg/mL gentamycin-sulphate (Life Technologies), and seeded at a density of 1.5×10^4 cells/well in a 24-well plate 24 h prior to transfection. Plasmids pcDNA3.1- μ NS N-FLAG and pcDNA3.1- λ 1 N-HA were transfected using Lipofectamine LTX reagent (Life Technologies) according to the manufacturer's instructions. The cells were fixed and stained 48 h post-transfection with an Intracellular Fixation and Permeabilization Buffer Set (eBioscience, San Diego, CA, USA) following the manufacturer's protocol. Antisera against μ NS (1:1000) and λ 1 (1:500); secondary antibody against rabbit IgG conjugated with Alexa Fluor 488 (Life Technologies) and Hoechst trihydrochloride trihydrate (Life Technologies) were used for staining. Images were captured on an inverted fluorescence microscope (Olympus IX81). Transfected EPC cells were also used to further verify anti- μ NS and anti- λ 1 in WB. A total of 3×5 million EPC cells were pelleted by centrifugation, resuspended in 100 μ L Ingenio Electroporation Solution (Mirus, Madison, WI, USA) and transfected with 4 μ g pcDNA3.1 μ NS N-FLAG or pcDNA3.1 λ 1 N-HA. The transfected cells were transferred to 75 cm² culture flasks containing 20 mL pre-equilibrated L-15 growth medium (described above) and collected 72 h post-transfection. The cell pellets were lysed in Nonidet-P40 lysis buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA) containing Complete ultra mini protease inhibitor cocktail (Roche, Mannheim, Germany). The mix was incubated on ice for 30 min, and then centrifuged at $5000 \times g$ for 5 min at 4 °C. The supernatant was mixed with Sample Buffer (Bio-Rad) and Reducing Agent (Bio-Rad), denatured for 5 min at 95 °C and run in SDS-PAGE, using 4%–12% Bis-Tris Criterion XT gel (Bio-Rad). Magic MarkTM XP Standard (Invitrogen) was used as a molecular size marker. Following SDS-PAGE, the proteins were blotted onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) and anti- μ NS and anti- λ 1 were used as primary antibodies and anti-Rabbit IgG-HRP (GE Healthcare, Buckinghamshire, UK) as secondary antibody. Protein bands were detected by chemiluminescence (Amersham ECL Plus, GE Healthcare).

2.6. Experimental Challenge of Salmon

A cohabitation challenge experiment was performed at VESO Vikan aquatic research facility, (Vikan, Norway). The fish had an average weight of 30 grams at the onset of the experiment with a maximum stocking density of 80 kg/m³, and were kept in 0.4 m³ tanks supplied with filtered and UV-radiated fresh water, 12 °C \pm 1 °C with a 12 h light/12 h dark regime. Water discharge of the tanks was provided by a tube overflow system with 7.2 L/min flow rate. The fish were acclimatized for two weeks prior to challenge, fed according to standard procedures and anesthetized by bath immersion (2–5 min) in benzocaine chloride (0.5 g/10 L water, Apotekproduksjon AS, Oslo, Norway) before handling. Briefly, the experimental study included one group of shedder fish (50%) marked at the time of PRV-injection by cutting off the adipose fin and one naïve cohabitant group (50%). The PRV inoculum was prepared from a batch of pooled heparinized blood samples from a previous PRV challenge experiment [19].

On day 0 of the challenge, the heparinized blood was diluted 1:2 in PBS and 0.1 mL of the inoculum was intraperitoneal (i.p.) injected into the shedders. The inoculum was confirmed negative for salmon viruses such as infectious pancreatic necrosis virus (IPNV), infectious salmon anemia virus

(ISAV), salmonid alphavirus (SAV) and piscine myocarditis virus (PMCV) by reverse transcription quantitative PCR (RT-qPCR). Samples from six fish were collected before initiation of the experiment to provide time-0 uninfected control material for protein assays. Heparinized blood was collected from six cohabitant fish at each sampling point; 3, 4, 5, 6, 7 and 8 weeks post challenge (wpc). In addition, a second cohabitation challenge experiment lasting 10 weeks was performed at the same facility following a similar experimental design. In this study, six fish sampled prior to PRV challenge were used to provide uninfected control material for protein and RT-qPCR assays, and heparinized blood was collected from six cohabitant fish at 4, 6, 8 and 10 wpc. The second challenge experiment was otherwise performed under the same conditions as the first experiment. Both experiments were approved by the Norwegian Animal Research Authority and followed the European Union Directive 2010/63/EU for animal experiments.

2.7. RNA Isolation and Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from 20 μ L heparinized blood homogenized in 650 μ L QIAzol Lysis Reagent (Qiagen, Hilden, Germany) using 5 mm steel beads, TissueLyser II (Qiagen) and RNeasy Mini spin column (Qiagen) as recommended by the manufacturer. RNA was quantified using a NanoDrop, ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The Qiagen OneStep kit (Qiagen) was used for RT-qPCR with a standard input of 100 ng (5 μ L of 20 ng/ μ L) of the isolated total RNA per reaction in a total reaction volume of 12.5 μ L. The template RNA was denatured at 95 °C for 5 min prior to RT-qPCR targeting PRV gene segments S1, M2 and M3. The following conditions were used for S1: 400 nM primer, 300 nM probe, 400 nM dNTPs, 1.26 mM MgCl₂, 1:100 RNase Out (Invitrogen) and 1 \times ROX reference dye with the following cycle parameters: 30 min at 50 °C, 15 min at 94 °C, 40 cycles of 94 °C/15 s, 54 °C/30 s and 72 °C/15 s in an AriaMx (Agilent, Santa Clara, CA, USA). Similar conditions and cycle parameters were also used targeting M2 and M3, although primer concentration was adjusted to 600 nM and annealing temperature to 58 °C. All samples were run in duplicates, and a sample was defined as positive if both parallel samples had a Ct <35. The fluorescence threshold for S1, M2 and M3 was set at Δ Rn 0.261, 0.028 and 0.021, respectively. The primers and probes are listed in Table S1. For analysis of antiviral gene expression, cDNA was prepared from 500 ng RNA using the QuantiTect reverse transcription kit with gDNA elimination (Qiagen) following the instructions from the manufacturer. Quantitative PCR was performed in triplets on 384-well plates using cDNA corresponding to 5 ng RNA in a total volume of 10 μ L per parallel, SsoAdvanced™ Universal SYBR® Green Supermix, and 500 nM forward and reverse primers (Table S2). The qPCRs were run for 40 cycles of 94 °C/15 s and 60 °C/30 s. All samples in the sample set were analyzed on the same plate using the same fluorescence threshold, and the cut-off value was set to Ct 37. The specificity of the SYBR green assays was confirmed by melting point analysis. Levels of Elongation factor (EF1 α) mRNA were used for normalization of all assays by the $\Delta\Delta$ Ct method.

2.8. Flow Cytometry

Samples consisting of 1.25 μ L heparinized blood (diluted 1:20 in PBS) from each of the cohabitant fish in the first challenge experiment were plated into 96-well plates for intracellular staining as previously described [19] using anti- μ NS and anti- σ 1 [4]. The corresponding zero serum, anti- μ NS Zero and anti- σ 1 Zero [4] were used as negative controls for background staining. Samples originating from 5 and 8 wpc were fixed, stained and analyzed immediately, while samples from 4 and 7 wpc were fixed and stored for one week and samples from 0, 3 and 6 wpc were fixed and stored for two weeks in flowbuffer (PBS, 1% BSA, 0.05% azide) before analysis. The cells were analyzed on a Gallios Flow Cytometer (Beckman Coulter, Miami, FL, USA), counting 50,000 cells per sample, and the data were analyzed using the Kaluza software (Becton Dickinson). Cells were gated according to size and granularity to include only intact cells and samples from 0 wpc were used as negative controls. Due to slight variation in background staining, the flow charts were gated individually to discriminate between negative and positive peaks.

2.9. Immunofluorescence Microscopy

Following flow cytometry analysis, the cells were prepared for immunofluorescence microscopy. The nuclei were stained with Hoechst trihydrochloride trihydrate (Life Technologies) and the cells were mounted to glass slides using Fluoroshield (Sigma-Aldrich, St. Louis, MO, USA) and cover slips. Images were captured on an inverted fluorescence microscope (Olympus IX81).

2.10. Transmission Electron Microscopy (TEM)

Samples consisting of 20 μ L heparinized blood from each cohabitant fish in the first experimental challenge were diluted in 1 mL PBS, centrifuged at $1000\times g$ for 5 min at 4 °C, washed twice in PBS and fixed in 3% glutaraldehyde overnight at 4 °C. All samples were further washed twice in PBS and prepared for transmission electron microscopy (TEM) as described earlier [19]. The sections were examined in a FEI MORGAGNI 268, and photographs were recorded using a VELETA camera.

2.11. Western Blotting (WB)

Heparinized blood from each cohabitant fish in the first challenge experiment was analyzed separately and as pooled samples from the different time-points. The samples were centrifuged at $5000\times g$ and the blood pellets were lysed in Nonidet-P40 lysis buffer containing Complete ultra mini protease inhibitor cocktail and prepared for WB as described above. Anti- μ NS (1:1000), anti- μ 1C (1:500) [4], anti- σ 1 (1:1000) [4], anti- σ 3 (1:500) [2] and anti- λ 1 (1:500) were used as primary antisera. Rabbit Anti-Actin (Sigma-Aldrich, St. Louis, MO, USA) was used to standardize the blots and Anti-Rabbit IgG-HRP (GE Healthcare) was used as secondary antibody. Blood collected at 0 wpc was used as negative control. In addition, heparinized blood from six of the cohabitant fish sampled at 0, 4, 6, 8 and 10 wpc in the second challenge experiment were prepared and analyzed in the same manner.

2.12. Immunoprecipitation (IP)

Blood from six cohabitants in the first challenge experiment sampled at 4, 5 and 8 wpc were pooled and lysed in Nonidet-P40 lysis buffer containing Complete ultra mini protease inhibitor cocktail as described above. The supernatants were transferred to new tubes and added anti- μ NS or anti- μ 1C (1:50) and incubated at 4 °C overnight with rotation. The Immunoprecipitation Kit Dynabeads Protein G (Novex, Life Technologies) was used for protein extraction and the beads were prepared according to the manufacturer's protocol. The cell-lysate-antibody mixtures were mixed with the protein G-coated beads and incubated 2 h at 4 °C. The beads-antibody-protein complexes were washed according to the manufacturer's protocol and run in SDS-PAGE. The SDS-gel was blotted onto PVDF membranes (Bio-Rad) and the proteins were detected using anti- μ NS, anti- μ 1C [4], anti- σ 1 [4], anti- σ 3 [2] and anti- λ 1.

2.13. Liquid Chromatography–Mass Spectrometry (LC–MS)

Five and three fragments immunoprecipitated with anti- μ NS (4 and 5 wpc) and anti- μ 1C (5 wpc), respectively, that were not observed at 0 wpc, were excised and in-gel digested with 0.1 μ g of trypsin in 20 μ L of 50 mM ammonium bicarbonate, pH 7.8 for 16 h at 37 °C (Promega, Madison, WI, USA). The peptides were purified with μ -C18 ZipTips (Millipore, Billerica, MA, USA), and analyzed using an Ultimate 3000 nano-UHPLC system (Dionex, Sunnyvale, CA, USA) connected to a Q Exactive mass spectrometer (ThermoElectron, Bremen, Germany). Liquid chromatography and mass spectrometry was performed as previously described [37]. Data were acquired using Xcalibur v2.5.5 and raw files were processed to generate peak list in Mascot generic format (*.mgf) using ProteoWizard release (Version 3.0.331). Database searches were performed using Mascot (Version 2.4.0) against the protein sequences of λ 1, λ 2, λ 3, μ NS, μ 1, μ 2, σ NS, σ 1, σ 2 and σ 3 assuming the digestion enzyme trypsin and semi-trypsin, at a maximum of one missed cleavage site, fragment ion mass tolerance of 0.05 Da, parent ion tolerance of 10 ppm and oxidation of methionines, propionamidylation of cysteines, acetylation of

the protein N-terminus as variable modifications. Scaffold 4.4.8 (Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications.

2.14. Computational Analysis

Theoretical molecular weights for proteins were calculated using the Compute pI/Mw tool [38]. PSI-blast based secondary structure PREDiction (PSIPRED; Version 3.3) was used to predict protein secondary structure [39].

2.15. Statistical Analysis

Differences in gene expression levels of innate antiviral genes was analyzed using one-way Anova with Tukey's multiple comparison test. Correlation analysis between PRV S1/M3 RNA levels and antiviral and immune gene expression were performed using nonparametric Spearman correlation.

3. Results

3.1. Viral RNA Load in Blood Cells

RT-qPCR targeting PRV genomic segments S1, M2 and M3 revealed high viral RNA loads in blood cells from 3 to 8 wpc (Figure 1). RNA from segments S1, M2 and M3 were first detected at 3 wpc and peaked at 5 wpc with mean Ct-values of 17.2 (± 0.4), 14.5 (± 0.3) and 14.6 (± 0.4). From 5 wpc, the S1 RNA load decreased, and by 8 wpc the mean Ct-value was 26.4 (± 0.6). However, a similar decrease was not observed for the M2 and M3 RNAs, and by 8 wpc mean Ct-values for these genomic segments were 17.4 (± 0.5) and 17.7 (± 0.4), respectively. RT-qPCR targeting genomic segment S1 in blood from six fish sampled at 0, 4, 6, 8 and 10 wpc in the second challenge experiment was also performed and gave similar results (Figure S2).

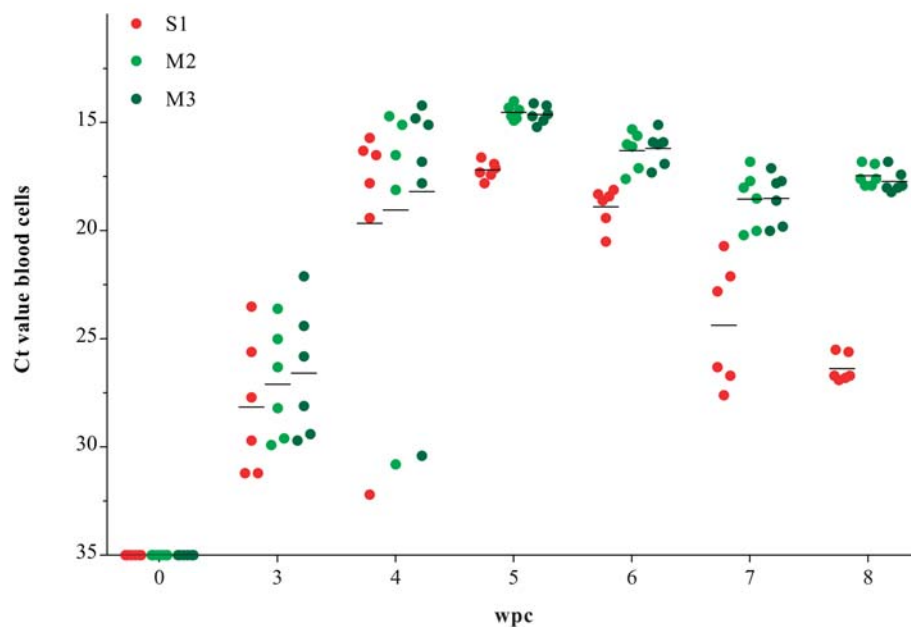


Figure 1. *Piscine orthoreovirus* (PRV) RNA load in blood cells. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) of PRV gene segments S1, M2 and M3 in blood cells from cohabitant fish. Individual (dots) and mean (line) Ct-values, $n = 6$ per time-point. wpc = weeks post challenge.

3.2. Expression of Innate Antiviral Genes in PRV Infected Blood Cells

The innate antiviral immune response in blood following PRV infection was studied by RT-qPCR targeting Atlantic salmon type I interferon (IFN α), viperin, interferon-stimulated gene 15 (ISG15), dsRNA-activated protein kinase (PKR) and IFN γ . All innate antiviral genes analyzed were statistically significantly upregulated during the peak phase of PRV infection from 4 to 6 wpc, increasing 5- to 20-fold compared to the level at 3 wpc (Figure 2a, Figure S3). The Ct values for S1 and M3 RNA correlated with the relative levels of gene expression for all innate antiviral genes, but not for the T-cell marker genes CD4 and CD8 (Figure 2b). When comparing the early phase up to the peak of infection (3–5 wpc) with the later phase (6–8 wpc), S1 RNA was correlated with the innate antiviral response in both phases, whereas M3 only showed significant correlation in the early phase (Figure 2b). EF1 α were stably expressed during PRV infection and were used for normalization of all other assays by the $\Delta\Delta$ Ct method (Figure S4) [15,40].

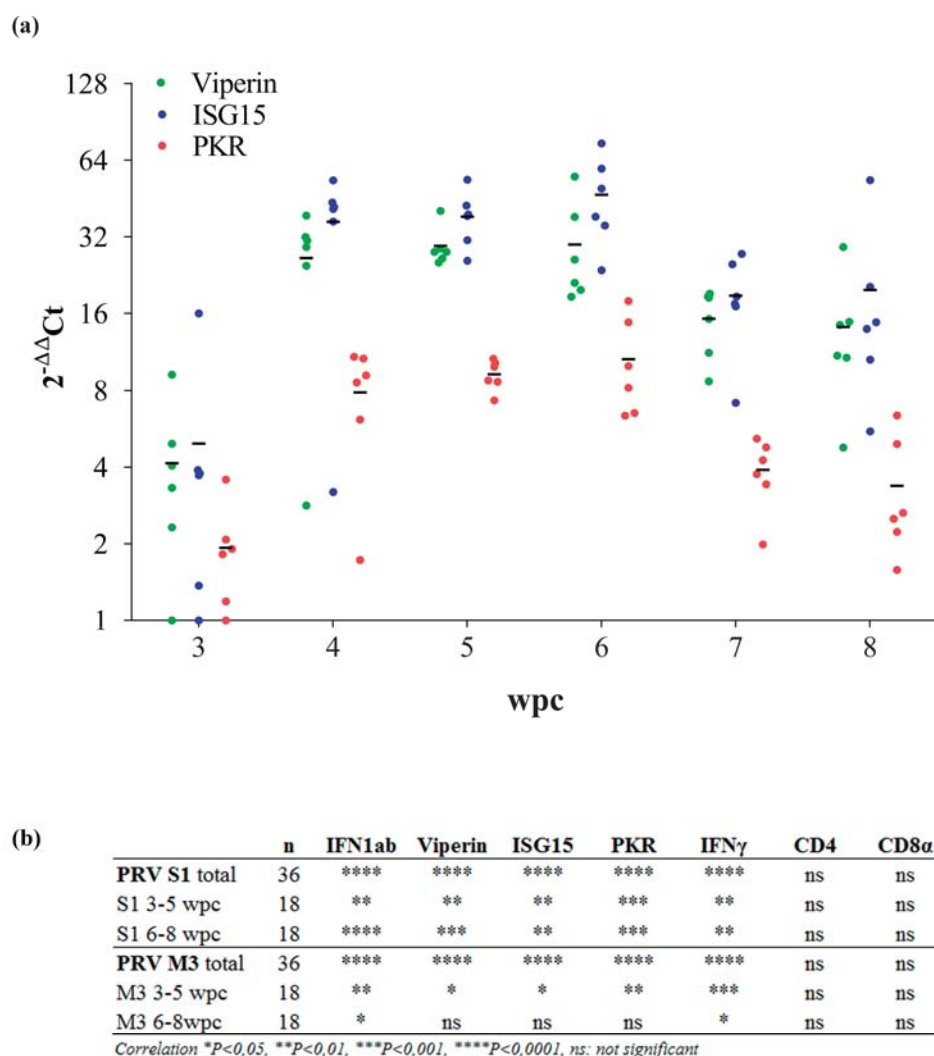


Figure 2. Expression of immune genes in blood cells. (a) Immune genes were assayed at 3–8 wpc by RT-qPCR in blood cells from cohabitant fish ($n = 6$ per time point). Data are normalized against EF1 α and the lowest Δ Ct level at 3 wpc ($n = 6$), and $2^{-\Delta\Delta$ Ct values are calculated. Mean relative expression is indicated. ISG = interferon-stimulated gene, PKR = double-stranded RNA (dsRNA)-activated protein kinase; (b) Correlation between Ct values for S1/M3 RNA and relative levels of antiviral gene expression for a set of immune genes.

3.3. Flow Cytometry Indicates a Transient Peak in Blood Cells

Blood cells stained intracellularly with anti- μ NS and anti- σ 1 were analyzed by flow cytometry (Figure 3a, Figure S5). A PRV positive population of blood cells was observed from 4 wpc as a marked shift in the histograms compared to negative samples. Five out of six fish were positive for μ NS by flow cytometry at 4 wpc, consistent with the RT-qPCR data where the positive fish had lower Ct-values (18.2 ± 5.6) compared to the negative fish (30.4). At 5 wpc, the PRV positive blood cell population decreased, but was still visible for all individuals. From 6 wpc and onwards, no PRV-positive cell populations were observed. The pattern for σ 1 positive cells was similar to that described for μ NS.

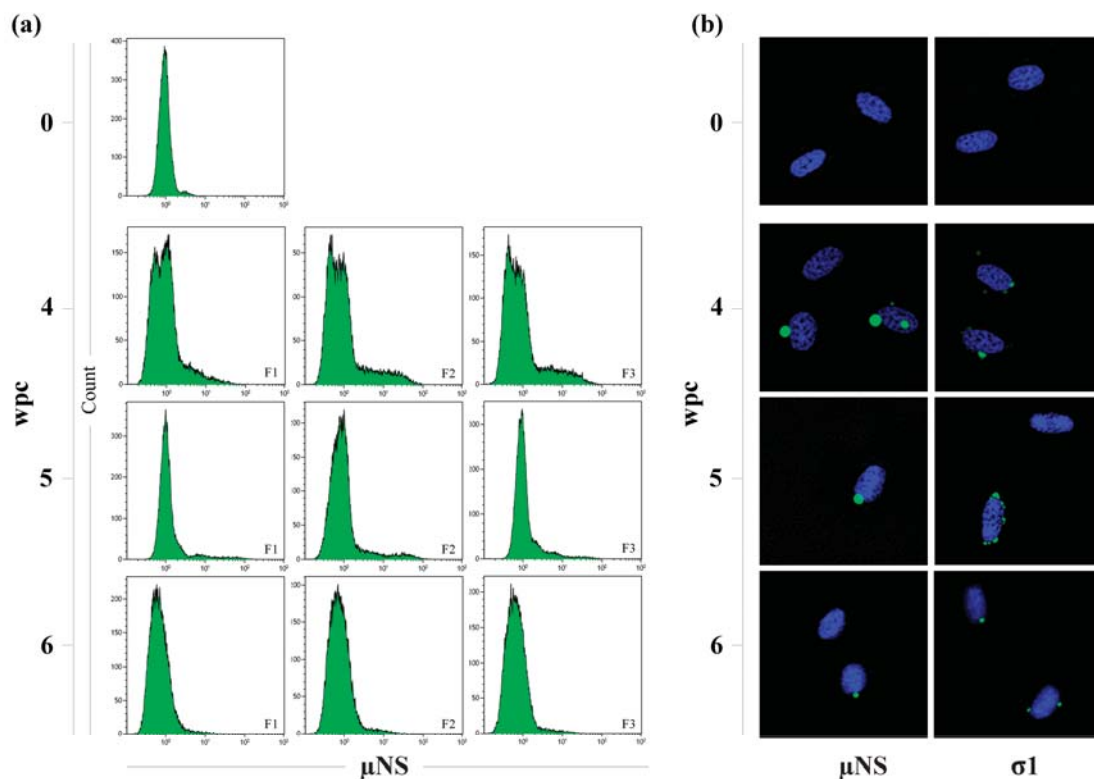


Figure 3. Presence of PRV μ NS and σ 1 in blood cells. (a) Intracellular staining of μ NS in blood cells analyzed by flow cytometry from three cohabitant fish sampled at 4, 5 and 6 wpc. The negative control staining is one fish sampled at 0 wpc. A total of 50,000 cells were counted per sample and 30,000 were gated for analysis; (b) Fluorescent labeling of μ NS (left) and σ 1 (right) displaying viral factory-like inclusions (green) in infected red blood cells sampled 0 (negative control), 4, 5 and 6 wpc. The nuclei were stained with Hoechst (blue).

3.4. Viral Factories Observed in Blood Cells

Both μ NS and σ 1 were detected by immunofluorescence as cytoplasmic globular inclusions in erythrocytes at 4, 5 and 6 wpc (Figure 3b). The inclusions varied in both size and number. At 4 and 5 wpc, they were predominantly large and perinuclear. Inclusions stained with anti- σ 1 were generally smaller and more variable in size than those stained with anti- μ NS. At 6 wpc, the number and size of the inclusions were considerably reduced and at 7 wpc and onward no inclusions were detected. These findings correlated with the results obtained from flow cytometry.

3.5. TEM of PRV Infected Blood Cells

TEM of PRV infected blood cells sampled at 0, 4, 5 and 6 wpc are shown in Figure 4. The control cells (0 wpc) contained circular cytoplasmic vesicles (200–500 nm) that were apparently devoid

of specific content. In addition, a few control cells contained lamellar structures up to 300 nm in size. At 4 wpc, lamellar structures were frequent and a few large cytoplasmic inclusions (~800 nm) containing particles with reovirus-like morphology were observed. The viral particles were naked with an electron dense core that resembled previous TEM descriptions of PRV [19]. At 5 wpc, several small (200–500 nm) and large (~800 nm) cytoplasmic inclusions containing reovirus-like particles were detected. The larger inclusions contained a mixture of reovirus-like particles and lamellar structures, some enclosed within membrane-like structures. At 6 wpc, large inclusions were frequent, but only a few contained viral particles.

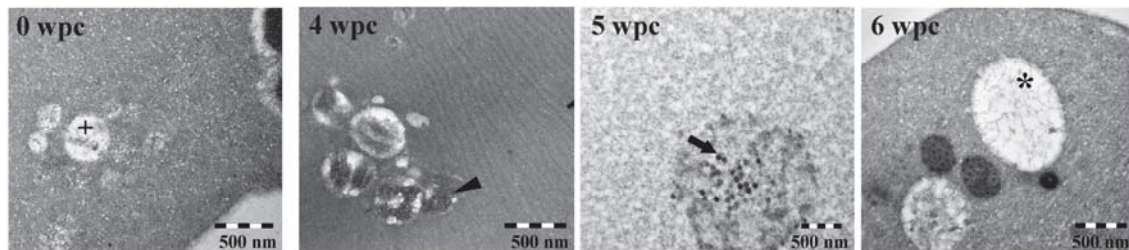


Figure 4. Transmission electron microscopy (TEM) of blood cells. PRV-infected red blood cells sampled at 0 (negative control), 4, 5 and 6 wpc show small empty vesicles (cross), lamellar structures (arrowhead), reovirus-like particles (arrow) and large empty inclusions (star).

3.6. μ NS Protein Expression in Individual Fish Correlate with viral RNA only during the Acute Phase of Infection

Blood cells from six fish sampled at 3, 4, 5 and 6 wpc were analyzed by WB using anti- μ NS and compared to Ct-values targeting the corresponding genomic segment M3 of the same samples (Figure 5). No fish were positive by WB at 3 wpc, while five samples at 4 wpc demonstrated bands at molecular weight (MW) 83.5 (putative full-length μ NS) and 70 kDa. The Ct-values from the same samples corresponded to the positive staining of the putative full-length μ NS bands. Fish 6 at 4 wpc, was negative for μ NS by WB; this individual also displayed a higher Ct-value (30.4) than the other cohabitants. The amount of μ NS decreased markedly from 4 to 5 wpc, and the 70 kDa band was barely detectable at 5 wpc. At 6 wpc, the μ NS protein was non-detectable by WB in fish 1, 5 and 6, and only barely detectable in the remaining fish. Although the μ NS protein level decreased below the detection limit for WB, the corresponding viral RNA levels (genomic segment M3) remained high throughout the challenge. Thus, μ NS protein and M3 RNA levels only correlated at 4 wpc.

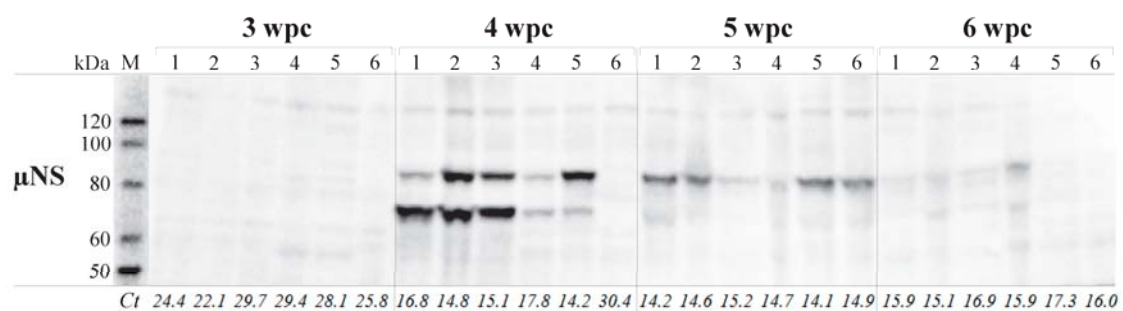


Figure 5. Detection of PRV uNS protein in blood cells compared to viral RNA load. Blood cells from 3, 4, 5 and 6 wpc ($n = 6$) analyzed for μ NS by Western blotting. Ct-values for gene segment M3 (μ NS) from the same samples are shown below each lane. M = molecular weight standard; Lane 1–6 refers to individual fish (1–6) per time point.

3.7. PRV Protein Levels Display a Transient Peak in Blood Cells

The load of structural proteins $\lambda 1$, $\mu 1$, $\sigma 1$ and $\sigma 3$, and the non-structural protein μNS , displayed a similar transient peak at 4–6 wpc in blood cells (Figure 6). All five proteins appeared at 4 wpc and were non-detectable at 7 wpc. In addition to the putative full-length μNS , a band with the MW of about 70 kDa was observed at 4 wpc, consistent with findings from individual fish (Figure 5). The putative full-length $\mu 1$ protein (74.2 kDa) was detected at 4 wpc. However, at 5 wpc, this band was not present but replaced by three bands of approximately 70 kDa, 37 kDa and 32 kDa in size. At 7 and 8 wpc, only one band of approximately 35 kDa was detected. The same staining patterns for the $\lambda 1$, μNS , $\mu 1$, $\sigma 1$ and $\sigma 3$ proteins were observed when blood from the second challenge experiment was analyzed (Figure S6).

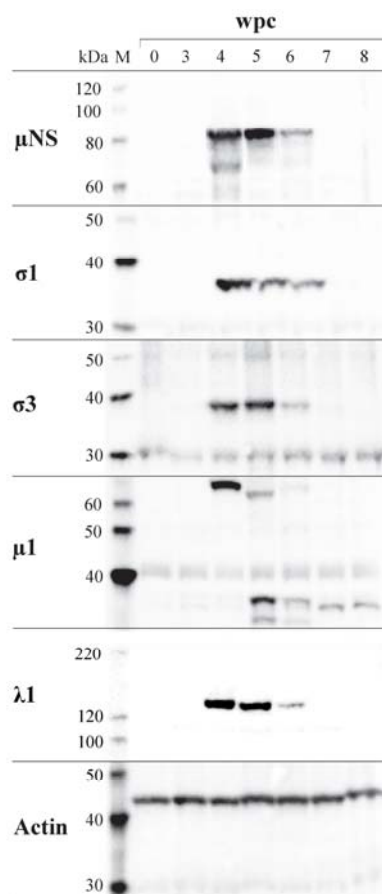


Figure 6. Presence of PRV proteins in blood cells. Pooled blood cell samples ($n = 6$) from each week were analyzed by Western blotting, targeting μNS , $\sigma 1$, $\sigma 3$, $\mu 1$ and $\lambda 1$. M = molecular weight standard. Actin was used as control for protein load.

3.8. PRV Proteins Interact with μNS

Interaction between μNS and other PRV proteins was studied by IP and WB (Figure 7). At 4 wpc, μNS was detected as a 70 kDa protein and at the same time-point the structural proteins $\lambda 1$, $\mu 1$, $\sigma 1$ and $\sigma 3$ were co-immunoprecipitated. At 5 wpc, μNS was detected in three different sizes ranging from 70 kDa to 83.5 kDa (putative full-length μNS). However, the only structural proteins co-immunoprecipitating with μNS at 5 wpc were $\sigma 3$ and the 35 kDa fragment of $\mu 1$ (see above). Interactions between μNS and other viral proteins were also investigated by liquid chromatography–mass spectrometry (LC–MS; Table 1) and peptides corresponding to $\lambda 1$, $\lambda 2$, $\lambda 3$, μNS , $\mu 1$, σNS and $\sigma 1$ were identified.

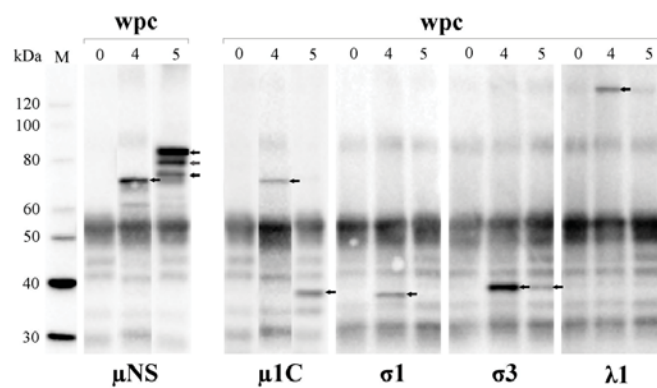


Figure 7. μ NS interacts with multiple PRV proteins. Pooled blood cell lysate ($n = 6$) immunoprecipitated with μ NS-antiserum, followed by Western blotting with primary antibodies detecting μ NS, μ 1C, σ 1, σ 3 and λ 1 (arrows). M = molecular weight standard.

Table 1. Identified *piscine orthoreovirus* (PRV) peptides following immunoprecipitation with anti- μ NS and mass spectrometry (MS).

* Band Excised from SDS-PAGE (kDa)	Identified PRV Proteins	Unique Peptides	Theoretical PRV Protein Size (kDa)
140 (5 wpc)	μ NS	1	83.5
130 (5 wpc)	λ 3	2	144.5
	λ 2	7	143.7
	λ 1	14	141.5
	μ NS	9	83.5
	σ 1	1	34.6
80 (5 wpc)	λ 1	11	141.5
	μ NS	24	83.5
70 (4 wpc)	μ NS	16	83.5
35 (5 wpc)	μ NS	4	83.5
	δ^+	3	37.7
	σ NS	1	39.1
	σ 1	2	34.6

* Approximate size of proteins excised from bands following IP with anti- μ NS antisera at four and five weeks post challenge (wpc). † Proteolytic fragment of μ 1 proposed in the present work.

3.9. μ NS Exists in Two Forms

WB of infected blood cells consistently produced two μ NS bands of approximately 83.5 and 70 kDa (Figures 5 and 6). Due to the presence of two translation initiation sites in MRV segment M3, the LC-MS data were analyzed to identify putative shorter variants of the PRV μ NS. The peptide distribution along the full-length μ NS sequence and their spectrum matches are shown in Figure S7a. The μ NS peptides and total spectrum matches obtained from the two bands are shown in Figure S7b. Several N-terminal μ NS peptides were identified from the 83.5 kDa band that were not observed in the 70 kDa band. Furthermore, the peptide spectrum matches from the 83.5 kDa and 70 kDa bands in the 200 amino acid N-terminus were 10 to 1, respectively. In contrast, for the remaining C-terminal μ NS sequence, the 83.5 kDa and 70 kDa bands produced similar or identical peptide spectrum matches, with a ratio of 66 to 63 (Figure S7). These results point to the presence of a second translation initiation site in the 5'-region of the μ NS ORF. Start sites at M₈₅, M₉₄, M₁₁₅ or M₁₆₉ would provide proteins with predicted sizes of 74.5, 73.6, 71.1 and 65.5 kDa, respectively. M₁₁₅ is the most likely candidate due to its size and presence in all PRV strains.

3.10. $\mu 1$ Has Two Putative Proteolytic Cleavage Sites

WB targeting the $\mu 1$ protein showed that the protein is present in different forms during infection. The putative full-length $\mu 1$ (74.1 kDa) was detected at 4 wpc (Figures 6 and 7). In contrast, smaller versions, with estimated sizes of 70, 37 and 32 kDa, replaced the full-length variant at 5 wpc (Figure 6). The three size variants from 5 wpc were subjected to LC–MS analysis (Figure S8). The 70 kDa band most likely represents $\mu 1C$ following pre-cleavage at $N_{42}P_{43}$ (MW 69.8 kDa). Of the fourteen peptide spectrum matches identified from the 70 kDa band, two were found to overlap $N_{42}P_{43}$ (Figure S8a). This is most likely due to carryover of slightly larger full-length $\mu 1$ (74.1 kDa) following gel excision. No peptides stretching N-terminal to $N_{42}P_{43}$ were identified from the 37 and 32 kDa bands. Additional semi-tryptic peptides, i.e., peptides generated by trypsin cleavage at one end but not the other, were identified from both the 37 and 32 kDa bands (Figure S8a). Among these is a peptide identified from the 32 kDa band harboring an N-terminal S_{388} . Cleavage of $\mu 1C$ at $F_{387}S_{388}$ would yield N- and C-terminal fragments of 37.7 kDa and 32.1 kDa, respectively. The distribution of peptide sequences and peptide spectrum matches provides support for proteolytic cleavage at or close to $F_{387}S_{388}$ (Figure S8). The results suggest that the 37 kDa and 32 kDa bands represent the PRV homologues of MRV $\mu 1$ fragments δ and ϕ , respectively. Besides the $\mu 1$ peptide sequences, peptides originating from other PRV proteins with sizes close to the sizes of the three excised fragments, were also identified. Peptide sequences matching $\lambda 1$ and μNS (one peptide spectrum match each) were identified from the 70 kDa band, sequences matching $\sigma 1$, $\sigma 3$ and σNS were identified from the 37 kDa band (2, 2 and 11 peptide spectrum matches, respectively) and $\sigma 2$ sequences were identified from the 32 kDa band (four peptide spectrum matches).

4. Discussion

Screening of farmed Atlantic salmon has indicated that PRV is ubiquitous in seawater and causes a persistent infection [9,11,41,42]. The study of PRV pathogenesis has been hampered by the lack of susceptible cell lines, and is currently dependent upon in vivo experiments. The fish in this experiment were challenged by cohabitation, i.e., through a natural transmission route. To ensure coordinated onset of infection, a high ratio of shedder fish was used. We found that PRV infection of salmon blood cells is acute and transient, with a peak lasting for 1–2 weeks under these experimental conditions.

Erythrocytes are major target cells for PRV [19]. Piscine erythrocytes are nucleated and contain the transcriptional and translational machinery enabling virus replication both in vivo and ex vivo [16,19]. We detected various PRV proteins in blood cells from 4 wpc, and the amount of protein was reduced at 6 wpc. Innate antiviral gene expression also peaked at 4–6 wpc and all selected genes were significantly induced during the peak period, in line with PRV protein production. In contrast to the transient peak displayed by PRV proteins, the viral RNA levels in blood cells persisted. The viral RNA level though, varied for the targeted genomic segments; the level of M2 ($\mu 1$) and M3 (μNS) remained high throughout the trial, while S1 ($\sigma 3$) transcripts decreased from 6 wpc. TEM analysis corresponded well with viral protein production, i.e., the lamellar structures observed at 4 wpc developed into inclusions containing reovirus-like particles at 5 wpc, while no virus particles could be observed at 7 wpc. The findings support PRV, causing an acute infection in blood cells where high PRV protein and particle production are sustained 1–2 weeks before the infection becomes persistent. Our study shows that, after the acute phase, the PRV RNA level as determined by RT-qPCR does not reflect the virus load in blood.

The salmon does not appear to be able to eliminate PRV. Challenge experiments have shown that PRV RNA can be detected at a steady level in heart and liver until 36 wpc (end of experiment) [41], and in blood for more than a year after challenge [9,19]. In an experiment where the infectious potential of persistently PRV infected Atlantic salmon was studied, sentinel fish were added at 59 wpc, but no transmission to the sentinel fish was observed [9]. This indicates that fish persistently infected with PRV do not continuously shed the virus. Viral persistence is common in fish and has been demonstrated for several RNA viruses [9,43–47]. The only PRV protein that could be detected after the peak of

virus protein production was a fragment of $\mu 1$, suggesting a possible role for this protein in persistent infection. In farmed salmon, where the size of the population in a net pen may exceed a hundred thousand individuals, and in the whole farm be more than a million fish, viral persistence in the population, but not necessarily in the individual, is also a critical parameter.

PRV infection in erythrocytes has previously been shown to induce expression of type I interferon and interferon-regulated genes [16,18]. In this study, the level of viral RNA correlated with the innate antiviral response in individual fish, with the exception of M3 expression after the virus peak (6–8 wpc). The continuous production of M3 RNA indicates that the innate antiviral immune response primarily inhibits virus replication post transcriptionally, which is in line with the functions of PKR and ISG15 on translation and protein modification, respectively [48,49].

Orthoreoviruses generate viral factories in the cytoplasm of infected cells [21,27,50–52], and PRV forms cytoplasmic globular viral factories resembling the structures produced by MRV T3D [16,19,31]. Viral factories are structures where virus replication and assembly occur, and thus where the viral proteins co-localize. The secluded nature of the viral factories modulates the level of the innate antiviral immune response. The orthoreoviral protein μ NS is orchestrating the construction of the factories and in this study and earlier studies we have found that $\lambda 1$, $\lambda 2$, $\lambda 3$, $\mu 1$, σ NS, $\sigma 1$, $\sigma 2$ and $\sigma 3$ interact with μ NS [31]. The $\sigma 3$ protein co-precipitated with μ NS but was not identified by MS, however WB can be more sensitive than LC–MS [52]. This suggests that μ NS interacts directly or indirectly with all three λ -proteins, the $\mu 1$ protein, and possibly all four σ -proteins. The μ NS protein was detected in different molecular sizes at specific time points. Further investigations led to the finding of four possible internal translation initiation sites in the μ NS gene. The M_{115} residue was determined to be the best candidate as M_{94} is not conserved among all PRV isolates, and M_{85} and M_{169} are unlikely due to the sizes of the proteins generated. Post-translational cleavage to generate μ NSC as shown for ARV μ NS cannot be excluded, although the specific proteolytic cleavage site in the ARV protein is not conserved in PRV [2,33,53]. The different μ NS size variants, i.e., full-length μ NS and the 70 kDa variant with putative translation initiation at M_{115} , may differ in their interactions with other PRV proteins. At 4 wpc, when only the 70 kDa variant of μ NS was detected following IP, all targeted structural proteins co-precipitated. However, at 5 wpc, when full-length μ NS was dominant, only the $\sigma 3$ protein and the assumed $\mu 1$ fragment δ co-precipitated. Studies previously performed on aquareoviruses and ARV indicate that recruitment of viral proteins into viral factories occurs in a predefined order through direct or indirect association with μ NS [50,54].

Four different molecular sizes of the $\mu 1$ protein were observed in the infected blood cells. Previous multiple sequence alignments of the $\mu 1$ amino acid sequence showed absolute conservation of the G2-myristoylation site and the autolytic $N_{42}P_{43}$ cleavage site, both regarded as crucial for reovirus $\mu 1$ -mediated membrane penetration [2]. The band observed at 4 wpc represents the full-length $\mu 1$ protein while the 70 kDa band at 5 wpc most likely represents $\mu 1C$.

Although peptides containing amino acid sequences overlapping the $N_{42}P_{43}$ site were observed from the 70 kDa band following LC–MS, peptides ending in P_{43} were present in equal amount. We conclude that the presence of the $N_{42}P_{43}$ overlapping peptides originate from carryover of the slightly larger full-length $\mu 1$ following gel excision. In addition, proteins can exhibit different abilities to separate in SDS-PAGE. This explains the presence of a minor fraction of peptides from the δ fragment (i.e., 37 kDa band) in the ϕ fragment (i.e., 32 kDa band) and vice versa. No peptide sequences overlapping $N_{42}P_{43}$ were identified from the 37 and 32 kDa bands. Rather, a higher number of peptides with an N-terminal P_{43} generated by non-tryptic cleavage were identified, providing additional support for cleavage at $N_{42}P_{43}$.

MRV $\mu 1$ contains a second cleavage site in its C-terminal region which, upon cleavage by exogenous proteases, generates the additional fragments δ and ϕ [55]. In the present study, we propose that the 37 kDa and 32 kDa bands represent the PRV homologues of the MRV δ and ϕ proteins. Hence, PRV ϕ contains a larger N-terminal portion of $\mu 1$ compared to MRV ϕ . Although there is only 28% identity at the amino acid level [2], the secondary structure of the PRV $\mu 1$ monomer predicted by

PSIPRED [39] (not shown) is very similar to that of MRV μ 1 [56]. This includes the helix-rich region in the C-terminal end [57], which for MRV largely constitutes the ϕ fragment shown to be crucial for membrane penetration, apoptosis induction and intracellular localization [57,58]. An interesting observation is that the three PRV μ 1 peptide sequences detected in the 35 kDa band following IP with anti- μ NS were all N-terminal to the proposed ϕ region, suggesting that μ NS-interacting sites on μ 1 may be located in the proposed δ region, between P₄₃ and F₃₈₇. From 7 wpc and onwards, the only PRV protein detected was a ~35 kDa protein which could represent the δ proteolytic fragment.

Production-related diseases are often multifactorial and the outcome of a PRV infection is influenced by viral strain, age of the fish, production and environmental factors. Recently, PRV was demonstrated to be the etiologic agent of EIBS, causing anemia and mass mortality in juvenile Coho salmon [7]. The level of anemia in EIBS corresponded well with the level of viral replication in blood and it is therefore tempting to suggest that EIBS is a consequence of acute PRV infection, i.e., the direct effect of virus PRV replication in erythrocytes. PRV is also the causative agent of HSMI [1,4], which appears 2–3 weeks after virus replication peaks in blood cells. The dominance of CD8 positive inflammatory cells found in the HSMI specific heart lesions indicates that immune mediated mechanisms are a major cause of the myocarditis.

In this study, we show that PRV infection has an acute phase in blood cells with high virus production before the infection subsides to a low persistent level. The continued transcription of viral RNA in the persistent phase suggests that the innate antiviral immune response may act to inhibit the virus infection post transcriptionally.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4915/9/3/49/s1, Figure S1: Specificity of μ NS and λ 1 antisera; Figure S2: PRV RNA load in blood cells (second challenge experiment); Figure S3: Expression of immune genes in blood cells; Figure S4: Expression of EF1 α during PRV infection; Figure S5: PRV σ 1 positive blood cells detected by flow cytometry; Figure S6: Presence of PRV proteins in blood cells (second challenge experiment); Figure S7: LC–MS analyses of PRV μ NS; Figure S8: LC–MS analyses of PRV μ 1; Table S1: Primers and probes used for construction of plasmids and expression of viral RNA levels; Table S2: Primers used for analysis of antiviral gene expression.

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Conflicts of Interest: The authors declare that no financial or commercial conflict of interest exists in relation to the content of this article.

References

1. Palacios, G.; Lovoll, M.; Tengs, T.; Hornig, M.; Hutchison, S.; Hui, J.; Kongtorp, R.T.; Savji, N.; Bussetti, A.V.; Solovyov, A.; et al. Heart and skeletal muscle inflammation of farmed salmon is associated with infection with a novel reovirus. *PLoS ONE* **2010**, *5*, e11487. [[CrossRef](#)] [[PubMed](#)]
2. Markussen, T.; Dahle, M.K.; Tengs, T.; Lovoll, M.; Finstad, O.W.; Wiik-Nielsen, C.R.; Grove, S.; Lauksund, S.; Robertsen, B.; Rimstad, E. Sequence analysis of the genome of piscine orthoreovirus (PRV) associated with heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (*Salmo salar*). *PLoS ONE* **2013**, *8*, e70075. [[CrossRef](#)]
3. Day, J.M. The diversity of the orthoreoviruses: Molecular taxonomy and phylogenetic divides. *Infect. Genet. Evol.* **2009**, *9*, 390–400. [[CrossRef](#)] [[PubMed](#)]

4. Finstad, O.W.; Falk, K.; Lovoll, M.; Evensen, O.; Rimstad, E. Immunohistochemical detection of piscine reovirus (PRV) in hearts of Atlantic salmon coincide with the course of heart and skeletal muscle inflammation (HSMI). *Vet. Res* **2012**, *43*, 27. [[CrossRef](#)] [[PubMed](#)]
5. Olsen, A.B.; Hjortaas, M.; Tengs, T.; Hellberg, H.; Johansen, R. First description of a new disease in Rainbow Trout (*Oncorhynchus mykiss* (Walbaum)) similar to Heart and skeletal muscle inflammation (HSMI) and detection of a gene sequence related to Piscine Orthoreovirus (PRV). *PLoS ONE* **2015**, *10*, e0131638. [[CrossRef](#)] [[PubMed](#)]
6. Sibley, S.D.; Finley, M.A.; Baker, B.B.; Puzach, C.; Armien, A.G.; Giehlbrock, D.; Goldberg, T.L. Novel reovirus associated with epidemic mortality in wild Largemouth Bass (*Micropterus salmoides*). *J. Gen. Virol.* **2016**, *97*, 2482–2487. [[PubMed](#)]
7. Takano, T.; Nawata, A.; Sakai, T.; Matsuyama, T.; Ito, T.; Kurita, J.; Terashima, S.; Yasuike, M.; Nakamura, Y.; Fujiwara, A.; et al. Full-Genome sequencing and confirmation of the causative agent of Erythrocytic inclusion body syndrome in Coho Salmon identifies a new type of Piscine Orthoreovirus. *PLoS ONE* **2016**, *11*, e0165424. [[CrossRef](#)] [[PubMed](#)]
8. Kibenge, M.J.; Iwamoto, T.; Wang, Y.; Morton, A.; Godoy, M.G.; Kibenge, F.S. Whole-genome analysis of piscine reovirus (PRV) shows PRV represents a new genus in family Reoviridae and its genome segment S1 sequences group it into two separate sub-genotypes. *Virol. J.* **2013**, *10*, 230. [[CrossRef](#)] [[PubMed](#)]
9. Garver, K.A.; Johnson, S.C.; Polinski, M.P.; Bradshaw, J.C.; Marty, G.D.; Snyman, H.N.; Morrison, D.B.; Richard, J. Piscine Orthoreovirus from Western North America is transmissible to Atlantic Salmon and Sockeye Salmon but fails to cause Heart and skeletal muscle inflammation. *PLoS ONE* **2016**, *11*, e0146229. [[CrossRef](#)] [[PubMed](#)]
10. Ferguson, H.W.; Kongtorp, R.T.; Taksdal, T.; Graham, D.; Falk, K. An outbreak of disease resembling heart and skeletal muscle inflammation in Scottish farmed salmon, *Salmo salar* L., with observations on myocardial regeneration. *J. Fish Dis.* **2005**, *28*, 119–123. [[CrossRef](#)] [[PubMed](#)]
11. Lovoll, M.; Alarcon, M.; Bang Jensen, B.; Taksdal, T.; Kristoffersen, A.B.; Tengs, T. Quantification of Piscine reovirus (PRV) at different stages of Atlantic salmon *Salmo salar* production. *Dis. Aquat. Organ.* **2012**, *99*, 7–12. [[CrossRef](#)] [[PubMed](#)]
12. Bjorgen, H.; Wessel, O.; Fjellidal, P.G.; Hansen, T.; Sveier, H.; Saebo, H.R.; Enger, K.B.; Monsen, E.; Kvellestad, A.; Rimstad, E.; et al. Piscine orthoreovirus (PRV) in red and melanised foci in white muscle of Atlantic salmon (*Salmo salar*). *Vet. Res.* **2015**, *46*, 89. [[CrossRef](#)] [[PubMed](#)]
13. Kongtorp, R.T.; Halse, M.; Taksdal, T.; Falk, K. Longitudinal study of a natural outbreak of heart and skeletal muscle inflammation in Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* **2006**, *29*, 233–244. [[CrossRef](#)] [[PubMed](#)]
14. Wiik-Nielsen, C.R.; Ski, P.M.; Aunsmo, A.; Lovoll, M. Prevalence of viral RNA from piscine reovirus and piscine myocarditis virus in Atlantic salmon, *Salmo salar* L., broodfish and progeny. *J. Fish Dis.* **2012**, *35*, 169–171. [[CrossRef](#)] [[PubMed](#)]
15. Johansen, L.H.; Dahle, M.K.; Wessel, O.; Timmerhaus, G.; Lovoll, M.; Rosaeg, M.; Jorgensen, S.M.; Rimstad, E.; Krasnov, A. Differences in gene expression in Atlantic salmon parr and smolt after challenge with *Piscine orthoreovirus* (PRV). *Mol. Immunol.* **2016**, *73*, 138–150. [[CrossRef](#)] [[PubMed](#)]
16. Wessel, O.; Olsen, C.M.; Rimstad, E.; Dahle, M.K. *Piscine orthoreovirus* (PRV) replicates in Atlantic salmon (*Salmo salar* L.) erythrocytes ex vivo. *Vet. Res.* **2015**, *46*, 26. [[CrossRef](#)] [[PubMed](#)]
17. Morera, D.; Roher, N.; Ribas, L.; Balasch, J.C.; Donate, C.; Callol, A.; Boltana, S.; Roberts, S.; Goetz, G.; Goetz, F.W.; et al. RNA-Seq reveals an integrated immune response in nucleated erythrocytes. *PLoS ONE* **2011**, *6*, e26998. [[CrossRef](#)] [[PubMed](#)]
18. Dahle, M.K.; Wessel, O.; Timmerhaus, G.; Nyman, I.B.; Jorgensen, S.M.; Rimstad, E.; Krasnov, A. Transcriptome analyses of Atlantic salmon (*Salmo salar* L.) erythrocytes infected with piscine orthoreovirus (PRV). *Fish Shellfish Immunol.* **2015**, *45*, 780–790. [[CrossRef](#)] [[PubMed](#)]
19. Finstad, O.W.; Dahle, M.K.; Lindholm, T.H.; Nyman, I.B.; Lovoll, M.; Wallace, C.; Olsen, C.M.; Storset, A.K.; Rimstad, E. *Piscine orthoreovirus* (PRV) infects Atlantic salmon erythrocytes. *Vet. Res.* **2014**, *45*, 35. [[CrossRef](#)] [[PubMed](#)]
20. Kongtorp, R.T.; Taksdal, T.; Lyngoy, A. Pathology of heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon *Salmo salar*. *Dis. Aquat. Organ.* **2004**, *59*, 217–224. [[CrossRef](#)] [[PubMed](#)]
21. Netherton, C.; Moffat, K.; Brooks, E.; Wileman, T. A guide to viral inclusions, membrane rearrangements, factories, and viroplasm produced during virus replication. *Adv. Virus Res.* **2007**, *70*, 101–182. [[PubMed](#)]

22. Knipe, D.M.; Howley, P.M. *Fields Virology*, 5th ed.; Wolters Kluwer/Lippincott Williams & Wilkins Health: Philadelphia, PA, USA, 2007; pp. 1854–1858.
23. Lee, P.W.; Hayes, E.C.; Joklik, W.K. Protein sigma 1 is the reovirus cell attachment protein. *Virology* **1981**, *108*, 156–163. [[CrossRef](#)]
24. Nibert, M.L.; Fields, B.N. A carboxy-terminal fragment of protein mu 1/mu 1C is present in infectious subviral particles of mammalian reoviruses and is proposed to have a role in penetration. *J. Virol.* **1992**, *66*, 6408–6418. [[PubMed](#)]
25. Thete, D.; Snyder, A.J.; Mainou, B.A.; Danthi, P. Reovirus mu1 protein affects infectivity by altering virus-receptor interactions. *J. Virol.* **2016**, *90*, 10951–10962. [[CrossRef](#)] [[PubMed](#)]
26. Becker, M.M.; Peters, T.R.; Dermody, T.S. Reovirus oNS and μ NS proteins form cytoplasmic inclusion structures in the absence of viral infection. *J. Virol.* **2003**, *77*, 5948–5963. [[CrossRef](#)] [[PubMed](#)]
27. Schiff, L.A.; Nibert, M.L.; Tyler, K.L. Orthoreoviruses and their replication. In *Fields virology*, 5th ed.; Knipe, D.M., Howley, P.M., Fields, B.N., Eds.; Wolters Kluwer/Lippincott Williams & Wilkins: Philadelphia, PA, USA, 2007; Volume 2, pp. 1853–1915.
28. Jayasuriya, A.K.; Nibert, M.L.; Fields, B.N. Complete nucleotide sequence of the M2 gene segment of reovirus type 3 dearing and analysis of its protein product mu 1. *Virology* **1988**, *163*, 591–602. [[CrossRef](#)]
29. Duncan, R. The low pH-dependent entry of avian reovirus is accompanied by two specific cleavages of the major outer capsid protein mu 2C. *Virology* **1996**, *219*, 179–189. [[CrossRef](#)] [[PubMed](#)]
30. Wiener, J.R.; Joklik, W.K. Evolution of reovirus genes: A comparison of serotype 1, 2, and 3 M2 genome segments, which encode the major structural capsid protein mu 1C. *Virology* **1988**, *163*, 603–613. [[CrossRef](#)]
31. Haatveit, H.M.; Nyman, I.B.; Markussen, T.; Wessel, O.; Dahle, M.K.; Rimstad, E. The non-structural protein μ NS of piscine orthoreovirus (PRV) forms viral factory-like structures. *Vet. Res.* **2016**, *47*, 5. [[CrossRef](#)] [[PubMed](#)]
32. Parker, J.S.; Broering, T.J.; Kim, J.; Higgins, D.E.; Nibert, M.L. Reovirus core protein mu2 determines the filamentous morphology of viral inclusion bodies by interacting with and stabilizing microtubules. *J. Virol.* **2002**, *76*, 4483–4496. [[CrossRef](#)] [[PubMed](#)]
33. Busch, L.K.; Rodriguez-Grille, J.; Casal, J.I.; Martinez-Costas, J.; Benavente, J. Avian and mammalian reoviruses use different molecular mechanisms to synthesize their microNS isoforms. *J. Gen. Virol.* **2011**, *92*, 2566–2574. [[CrossRef](#)] [[PubMed](#)]
34. Touris-Otero, F.; Martinez-Costas, J.; Vakharia, V.N.; Benavente, J. Avian reovirus nonstructural protein microNS forms viroplasm-like inclusions and recruits protein sigmaNS to these structures. *Virology* **2004**, *319*, 94–106. [[CrossRef](#)] [[PubMed](#)]
35. Wiener, J.R.; Bartlett, J.A.; Joklik, W.K. The sequences of reovirus serotype 3 genome segments M1 and M3 encoding the minor protein mu 2 and the major nonstructural protein mu NS, respectively. *Virology* **1989**, *169*, 293–304. [[CrossRef](#)]
36. McCutcheon, A.M.; Broering, T.J.; Nibert, M.L. Mammalian reovirus M3 gene sequences and conservation of coiled-coil motifs near the carboxyl terminus of the microNS protein. *Virology* **1999**, *264*, 16–24. [[CrossRef](#)] [[PubMed](#)]
37. Koehler, C.J.; Bollineni, R.C.; Thiede, B. Application of the half decimal place rule to increase the peptide identification rate. *Rapid Commun. Mass Spectrom.* **2016**, *31*, 227–233. [[CrossRef](#)] [[PubMed](#)]
38. ExPASy Bioinformatics Resource Portal. Available online: http://web.expasy.org/compute_pi/ (accessed on 1 April 2016).
39. UCL Department Of Computer Science. Available online: <http://bioinf.cs.ucl.ac.uk/psipred/> (accessed on 1 April 2016).
40. Su, J.; Zhang, R.; Dong, J.; Yang, C. Evaluation of internal control genes for qRT-PCR normalization in tissues and cell culture for antiviral studies of grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunol.* **2011**, *30*, 830–835. [[CrossRef](#)] [[PubMed](#)]
41. Lovoll, M.; Wiik-Nielsen, J.; Grove, S.; Wiik-Nielsen, C.R.; Kristoffersen, A.B.; Faller, R.; Poppe, T.; Jung, J.; Peadarallu, C.S.; Nederbragt, A.J.; et al. A novel totivirus and piscine reovirus (PRV) in Atlantic salmon (*Salmo salar*) with cardiomyopathy syndrome (CMS). *Virol. J.* **2010**, *7*, 309. [[CrossRef](#)] [[PubMed](#)]
42. Marty, G.D.; Morrison, D.B.; Bidulka, J.; Joseph, T.; Siah, A. Piscine reovirus in wild and farmed salmonids in British Columbia, Canada: 1974–2013. *J. Fish Dis.* **2015**, *38*, 713–728. [[CrossRef](#)] [[PubMed](#)]

43. Julin, K.; Johansen, L.H.; Sommer, A.I.; Jorgensen, J.B. Persistent infections with infectious pancreatic necrosis virus (IPNV) of different virulence in Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* **2015**, *38*, 1005–1019. [[CrossRef](#)] [[PubMed](#)]
44. Gjessing, M.C.; Kvellestad, A.; Ottesen, K.; Falk, K. Nodavirus provokes subclinical encephalitis and retinochoroiditis in adult farmed Atlantic cod, *Gadus morhua* L. *J. Fish Dis.* **2009**, *32*, 421–431. [[CrossRef](#)] [[PubMed](#)]
45. Amend, D.F. Detection and transmission of infectious hematopoietic necrosis virus in rainbow trout. *J. Wildl. Dis.* **1975**, *11*, 471–478. [[CrossRef](#)] [[PubMed](#)]
46. Neukirch, M. Demonstration of persistent viral haemorrhagic septicaemia (VHS) virus in rainbow trout after experimental waterborne infection. *Zentralbl Veterinarmed B* **1986**, *33*, 471–476. [[CrossRef](#)] [[PubMed](#)]
47. Hershberger, P.K.; Gregg, J.L.; Grady, C.A.; Taylor, L.; Winton, J.R. Chronic and persistent viral hemorrhagic septicemia virus infections in Pacific herring. *Dis. Aquat. Organ.* **2010**, *93*, 43–49. [[CrossRef](#)] [[PubMed](#)]
48. Dalet, A.; Gatti, E.; Pierre, P. Integration of PKR-dependent translation inhibition with innate immunity is required for a coordinated anti-viral response. *FEBS Lett.* **2015**, *589*, 1539–1545. [[CrossRef](#)] [[PubMed](#)]
49. Durfee, L.A.; Lyon, N.; Seo, K.; Huibregtse, J.M. The ISG15 conjugation system broadly targets newly synthesized proteins: Implications for the antiviral function of ISG15. *Mol. Cell* **2010**, *38*, 722–732. [[CrossRef](#)] [[PubMed](#)]
50. Touris-Otero, F.; Cortez-San Martin, M.; Martinez-Costas, J.; Benavente, J. Avian reovirus morphogenesis occurs within viral factories and begins with the selective recruitment of sigmaNS and lambdaA to microNS inclusions. *J. Mol. Biol.* **2004**, *341*, 361–374. [[CrossRef](#)] [[PubMed](#)]
51. Carroll, K.; Hastings, C.; Miller, C.L. Amino acids 78 and 79 of Mammalian Orthoreovirus protein microNS are necessary for stress granule localization, core protein lambda 2 interaction, and de novo virus replication. *Virology* **2014**, *448*, 133–145. [[CrossRef](#)] [[PubMed](#)]
52. Aebersold, R.; Burlingame, A.L.; Bradshaw, R.A. Western blots versus selected reaction monitoring assays: time to turn the tables? *Mol. Cell. Proteomics* **2013**, *12*, 2381–2382. [[CrossRef](#)] [[PubMed](#)]
53. Rodriguez-Grille, J.; Busch, L.K.; Martinez-Costas, J.; Benavente, J. Avian reovirus-triggered apoptosis enhances both virus spread and the processing of the viral nonstructural muNS protein. *Virology* **2014**, *462–463*, 49–59. [[CrossRef](#)] [[PubMed](#)]
54. Yan, L.; Zhang, J.; Guo, H.; Yan, S.; Chen, Q.; Zhang, F.; Fang, Q. Aquareovirus NS80 initiates efficient viral replication by retaining core proteins within replication-associated viral inclusion bodies. *PLoS ONE* **2015**, *10*, e0126127. [[CrossRef](#)] [[PubMed](#)]
55. Nibert, M.L.; Odegard, A.L.; Agosto, M.A.; Chandran, K.; Schiff, L.A. Putative autocleavage of reovirus mu1 protein in concert with outer-capsid disassembly and activation for membrane permeabilization. *J. Mol. Biol.* **2005**, *345*, 461–474. [[CrossRef](#)] [[PubMed](#)]
56. Liemann, S.; Chandran, K.; Baker, T.S.; Nibert, M.L.; Harrison, S.C. Structure of the reovirus membrane-penetration protein, Mu1, in a complex with its protector protein, Sigma3. *Cell* **2002**, *108*, 283–295. [[CrossRef](#)]
57. Coffey, C.M.; Sheh, A.; Kim, I.S.; Chandran, K.; Nibert, M.L.; Parker, J.S. Reovirus outer capsid protein micro1 induces apoptosis and associates with lipid droplets, endoplasmic reticulum, and mitochondria. *J. Virol.* **2006**, *80*, 8422–8438. [[CrossRef](#)] [[PubMed](#)]
58. Danthi, P.; Coffey, C.M.; Parker, J.S.; Abel, T.W.; Dermody, T.S. Independent regulation of reovirus membrane penetration and apoptosis by the mu1 phi domain. *PLoS Pathog.* **2008**, *4*, e1000248. [[CrossRef](#)] [[PubMed](#)]



III

DNA vaccine expressing the non-structural proteins of *Piscine orthoreovirus* induces moderate protection against heart and skeletal muscle inflammation in Atlantic salmon (*Salmo salar*)

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Abstract

Piscine orthoreovirus (PRV) is the causative agent of heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon (*Salmo salar*). HSMI causes significant economic losses to the salmon aquaculture industry, and there is currently no vaccine available. Like other orthoreoviruses, PRV structures its replication and assembly within cytoplasmic inclusions called viral factories, organized by the viral protein μ NS. The non-structural μ NS and σ NS proteins are only present in cells during replication of PRV and are not part of the viral particle. In two experimental vaccination trials in Atlantic salmon, using DNA vaccines expressing different combinations of PRV proteins, we have shown that expression of the non-structural proteins induce moderate protection against HSMI. In particular, expression of μ NS and σ NS combined with the cell attachment protein σ 1, induced a protective effect against HSMI.

Introduction

Aquaculture is fast-growing globally and expected to increase further to meet future food demand. Infectious diseases are intrinsic to large-scale monoculture farming and may threaten the sustainability of the aquaculture industry due to high procreation, rapid spread and lack of efficient prophylactic measures. Heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (*Salmo salar*) is caused by *Piscine orthoreovirus* (PRV) [1], and is one of the most prevalent viral diseases in Norwegian salmon aquaculture [2], also reported in Scotland, Chile and Canada [3-5]. The disease is mainly observed during the seawater grow-out phase and has a slow disease development [6]. The histopathological characteristics of HSMI are epi-, endo- and myocarditis, myocardial necrosis, myositis and necrosis of the red skeletal muscle [7, 8]. The accumulated mortality is moderate, ranging from negligible to 20%. The morbidity, on the other hand, i.e. the prevalence of histopathological changes typical of HSMI in a diseased population, is close to 100% [7]. HSMI leads to significant economic losses in Atlantic salmon aquaculture in Norway. Intervention by optimized management remains a challenge, as the knowledge of PRV transmission routes and effects of disinfection are limited.

PRV is present in farmed Atlantic salmon in Europe, North- and South America, and considered ubiquitous in the marine phase of Atlantic salmon farming. A virus closely related to PRV, named PRV-2, was demonstrated to be the etiological agent of erythrocytic inclusion body syndrome (EIBS) in juvenile Coho salmon (*Onchorhynchus kisutchi*), causing anemia and mass mortality [9]. Infection of farmed rainbow trout in fresh water by yet another PRV-like virus, has been associated with anemia and HSMI-like disease [10].

PRV is in the family *Reoviridae*, genus *Orthoreovirus*, containing ten double-stranded RNA (dsRNA) genome segments encapsulated in a double-shelled protein capsid [11, 12]. The genome segments encoding 10 -13 proteins are divided into three size classes; three large (L), three medium (M) and four small (S), expressing the λ , μ and σ proteins, respectively [12, 13]. Erythrocytes are major target cells for PRV and important for pathogenesis [14], but PRV also infects myocytes of the heart- and skeletal muscles [15]. Influx of inflammatory cells into heart and muscle, which commences 1-2 weeks after peak viral replication [16], has named the disease.

Cytoplasmic, globular inclusions that resemble orthoreoviral factories are formed in infected erythrocytes [14, 17]. The concentration of the viral products in temporary organelles, called viral factories, facilitates coordination of the replication, packaging and particle assembly, and provides protection from the cellular antiviral response [18]. As shown in *Mammalian orthoreovirus* (MRV) infections, viral factories are formed as small punctate structures throughout the cytoplasm early after infection, and grow in size becoming perinuclear as the infection progresses [19]. For both MRV and PRV, μ NS is the scaffolding protein that organizes these factories [20, 21].

As demonstrated for MRV, trimeric σ 1 proteins associates with λ 2 forming the cell attachment complex, and distinct domains of σ 1 are involved in binding to target cell receptors [22-27]. Following attachment to the cell, reoviruses are internalized by receptor-mediated endocytosis [28, 29]. Their release into the cytoplasm through the endosomal membrane is associated with removal of the outer capsid protein σ 3 and proteolytic cleavage of μ 1 [28, 30]. Further proteolytic cleavage after cytoplasmic translocation removes the remaining outer capsid proteins, generating transcriptionally active core particles [31]. For MRV it has been shown that monoclonal antibodies that interfere with cell attachment, endosomal release or viral uncoating, i.e. directed against outer capsid proteins σ 1, σ 3 and μ 1C, as well as core protein λ 2, can neutralize the virus [32].

PRV has so far resisted propagation in cell cultures, which has made production of inactivated whole-virus vaccines difficult. In experimental settings, DNA vaccination against viral diseases in salmonids such as viral haemorrhagic septicaemia (VHS), infectious salmon anemia (ISA) and pancreas disease (PD) have induced efficient protection [33-35]. A DNA vaccine against infectious hematopoietic necrosis (IHN) has been used in Canadian aquaculture since 2005 [36] and so far there has been no reports of IHN outbreaks in the vaccinated populations. Alphavirus replicon vectors, where the non-structural genes are retained and viral structural protein genes are exchanged with a gene of interest (GOI), i.e. vaccine antigen, have been developed from several different mammalian alphaviruses and represent efficient tools in recombinant vaccine development [37]. A salmonid alphavirus (pSAV) replicon vector, has been found to induce efficient protection against ISA and PD in experimental trials [33, 34, 38-40]. The generation of double-stranded RNA intermediates from such vectors are assumed to trigger a favorable immune response after vaccination [41].

The present study was conducted to examine whether DNA vaccines expressing PRV non-structural proteins, alone or in combination with structural PRV proteins, could induce protection against HSMI. Both pSAV replicon-driven PRV protein expression and conventional CMV promoter-driven PRV protein expression were tested. We hypothesized that expression of the virus factory assembly protein μ NS could provide an efficient trigger of the host immune response during a vaccination trial against HSMI.

Materials and methods

Plasmid constructs

The full-length open reading frames (ORFs) of PRV genes encoding λ 1, λ 2, λ 3, μ 1, μ 2, μ NS, σ 1, σ 2, σ 3 and σ NS, were amplified using PfuUltra II Fusion HS DNA polymerase (Agilent, Santa Clara, CA, USA) and cDNA prepared in an earlier study [21]. pSAV replicon vectors [33] expressing each of these ORFs individually, and the eukaryotic expression vector pcDNA3.1 (+) (Invitrogen) expressing PRV μ NS, σ NS, σ 1, σ 3 or enhanced Green fluorescent protein (EGFP) (control), were constructed. In short, the PCR amplicons of the ORFs were either cloned into the AgeI and AscI restriction sites of the pSAV replicon (thereby removing the EGFP of the original replicon construct), or into the XbaI restriction site of pcDNA3.1. Six additional plasmids containing an epitope tag fused to the gene of interest; pSAV/ σ NS N-MYC, pcDNA3.1/ σ NS N-MYC, pSAV/ σ 2 N-HA, pSAV/ μ 2 N-HA, pSAV/ λ 2 N-HA and pSAV/ λ 3 N-HA, were also constructed for expression analysis as described earlier [21]. Primer sequences are listed in Table S1. Sanger sequencing (GATC Biotech AG, Konstanz, Germany) verified all sequences.

Transfections of fish cells

CHSE-214 cells (ATCC CRL-1681, Chinook salmon embryo) were cultivated in Leibovitz L-15 medium (L15, Life Technologies, Carlsbad, USA) supplemented with 10 % heat inactivated fetal bovine serum (FBS, Life technologies), 2 mM L-glutamine, 0.04 mM mercaptoethanol and 0.05 mg/ml gentamycin-sulphate (Life Technologies). A total of 3 million CHSE cells were pelleted by centrifugation, resuspended in 100 μ L Ingenio Electroporation Solution (Mirus, Madison, WI, USA) and separately transfected with 3 μ g of each the plasmids expressing λ 1, λ 2, λ 3, μ 1, μ 2, μ NS, σ 1, σ 2, σ 3 or σ NS using the Amaxa T-20 program. The transfected cells were diluted in 1 mL pre-equilibrated L-15 growth medium and 100 μ L of the diluted cells was seeded onto gelatin-embedded cover slips (12 mm) in a 24-well plate for

expression analysis by immunofluorescence microscopy. Transfections with pSAV/EGFP and pcDNA3.1/EGFP constructs were used as positive expression controls.

Immunofluorescence microscopy

Transfected CHSE-214 cells were fixed and stained using an intracellular Fixation and Permabilization Buffer (eBioscience, San Diego, CA, USA). The cells were washed in Dulbecco's PBS (DPBS) with sodium azide. Intracellular fixation buffer was added before incubation with primary antibodies, anti- λ 1 (1:1000) [16], anti- μ 1C (1:500) [15], anti- μ NS (1:1000) [16], anti- σ 1 (1:1000) [15], anti- σ 3 (1:1000) [12], anti-myc (goat anti-myc antibody, Abcam; Cambridge, UK) or anti-HA (rabbit anti-HA antibody, Sigma-Aldrich; St Louis, MO, USA). Secondary antibodies were anti-rabbit immunoglobulin G (IgG) conjugated with Alexa Fluor 488 (Life Technologies, 1:400) or anti-goat IgG conjugated with Alexa Fluor 594 (Life Technologies, 1:400). Nuclear staining was performed with Hoechst trihydrochloride trihydrate stain solution (Life Technologies). The cover slips were mounted onto glass slides using Fluoroshield (Sigma-Aldrich) and images were captured on an inverted fluorescence microscope (Olympus IX81).

Vaccine preparations

The concentration of plasmid constructs were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and diluted in PBS to 1000 ng/ μ L. Samples for vaccination were prepared to contain 10 μ g of each plasmid construct in a total volume 50 μ L (Table 1). The samples were blinded before initiation of the challenge experiments.

Vaccination trials

Two cohabitant challenge experiments were performed at VESO Vikan aquatic research facility (Vikan, Norway) in order to evaluate the vaccine efficacy against HSMI following immunization with pSAV-based replicon vaccines and pcDNA3.1-based expression vaccines (Figure 1). The trials were performed using previously unvaccinated Atlantic salmon pre-smolts with an average weight of 30-40 g, confirmed free of common salmon pathogens. The fish were kept in a freshwater flow-through system (temperature: 12°C; oxygen: > 70%; pH 6.6-6.9), acclimatized for 1 week and starved 48 hours prior to vaccination. The fish were randomly selected for vaccination, anesthetized by bath immersion (2–5 min) in benzocaine chloride (0.5 g/10 L water, Apotekproduksjon AS, Oslo, Norway), labelled with passive

integrated transponder (PIT) tags (two weeks prior to vaccination) and intramuscularly (i.m.) injected with the vaccines or control substances. The challenges were performed in connection with transfer to seawater after a six week immunization- and smoltification/photoperiod manipulation. The shedders were i.p. injected with 0.1 mL of pooled heparinized blood samples from a previous PRV challenge experiment [14]. The inoculum was confirmed negative for the salmon viruses including infectious pancreatic necrosis virus (IPNV), infectious salmon anemia virus (ISAV), salmonid alphavirus (SAV) and piscine myocarditis virus (PMCV) by RT-qPCR. The fish were starved for 24 hours prior to challenge. The experiments were approved by the Norwegian Animal Research Authority and followed the European Union Directive 2010/63/EU for animal experiments.

In vaccination trial #I the fish were divided into seven groups, each containing 40 fish, and immunized by i.m. injection of 10 µg/50 µL per pSAV replicon based vaccine construct, pcDNA3.1 based vaccine construct or control pSAV/EGFP replicon (Table 1). The vaccination day was defined as day 0 of the experiment. Ten untreated fish were sampled as controls prior to the experiment. Another six fish per group were sampled two and six weeks after vaccination. Six weeks after vaccination, approximately 20 % PRV shedders were introduced to the challenge tank. The fish were observed daily and fed according to standard procedures. Six fish per group were sampled at 4 weeks post challenge (wpc), 6 wpc, 8 wpc and 10 wpc, and euthanized using an overdose of anesthetics.

In vaccination trial #II, the fish were divided into six groups, each containing 26 fish, and immunized by i.m. injection of 10 µg/50 µL per pcDNA3.1 construct, control construct (pcDNA3.1/EGFP) or PBS (Table 1). At 4 wpc, six fish from the PBS control group were sampled and analyzed for viral RNA loads in blood to determine suitable time points for the following two samplings, set to 6 and 8 wpc. Further, 12 fish per group were sampled at these two time-points before termination of the experiment. Heparinized blood, plasma and heart (stored in 4% formalin or RNAlater) were sampled from both challenge experiments.

RNA isolation and RT-qPCR

Total RNA was isolated from 20 µL heparinized blood homogenized in 650 µL QIAzol Lysis Reagent (Qiagen, Hilden, Germany) using 5 mm steel beads, TissueLyser II (Qiagen) and RNeasy Mini spin column (Qiagen) as recommended by the manufacturer. RNA quantification was performed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific,

Wilmington, DE, USA). For the plasma samples, a 10 μ L volume was diluted in PBS to 140 μ L and used in the Mini spin column (Qiagen), as recommended by the manufacturer. The Qiagen OneStep kit (Qiagen) was used for RT-qPCR with a standard input of 100 ng (5 μ L of 20 ng/ μ L) of the isolated total RNA per reaction. From the cell free plasma samples, 5 μ L input of total eluted RNA was used. The template RNA was denatured at 95°C for 5 min prior to RT-qPCR targeting PRV gene segment S1 (S1Fwd: 5'TGCGTCCTGCGTATGGCACC'3, S1Rev: 5'GGCTGGCATGCCCGAATAGCA'3 and S1probe: 5'-FAM-ATCACAACGCCTACCT'3-MGBNFQ) using the following conditions: 400 nM primer, 300 nM probe, 400 nM dNTPs, 1.26 mM MgCl₂, 1:100 RNase Out (Invitrogen) and 1 \times ROX reference dye. The cycling conditions were 50°C for 30 min and 94°C for 15 min, followed by 35 cycles of 94°C/15 sec, 54°C/30 sec and 72°C/15 sec in an AriaMx (Agilent, Santa Clara, CA, USA). All samples were run in duplicates, and a sample was defined as positive if both parallels produced a Ct value below 35.

Histopathological scoring

Sections for histopathology were processed and stained with hematoxylin and eosin following standard procedures. Individual fish from both vaccination trials were examined for heart lesions in consistence with HSMI, discriminating between epicardial and myocardial changes. The grade of changes was scored from 0 to 4 using criteria described in Table S2. The individual histopathological scores were used to calculate the mean score \pm SD at each time point of sampling (n = 6 or n = 12) for both epicardial and myocardial changes.

Statistical analyses

The PRV RT-qPCR results and the histopathology scores were analyzed statistically using the Mann Whitney compare ranks test due to the small sample sizes (n = 6/12). All statistical analysis described were performed with GraphPad Prism (GraphPad Software inc., USA) and p-values of $p \leq 0.05$ were considered as significant.

Results

Expression analysis

All plasmid constructs expressed PRV proteins as observed following transfection of CHSE-214 cells (Table S3). Expression of the μ NS and σ 1 proteins are presented in Figure 2. The μ NS protein formed small punctuate structures throughout the cytoplasm, while σ 1 had an

even, diffuse, distribution pattern. The pcDNA3.1 constructs yielded a higher number of positive cells (approximately 40 %), indicating better transfection efficacy, than the pSAV replicon construct (approximately 15 %), as estimated visually. For some of the pSAV constructs, only 5 - 10 % transfection efficacy were achieved (data not shown).

Vaccination trial #I

pcDNA3.1 expressing μ NS + σ NS + σ 1 significantly reduced PRV loads in blood

The mean PRV Ct-values in blood from all groups, including control group (pSAV/EGFP) in vaccination trial #I are shown in Figure S1. PRV was first detected by RT-qPCR in blood at 4 wpc in all groups. In the pSAV/EGFP control group, PRV RNA levels were high, peaking at 6 wpc with a mean Ct-value of 14.8 (\pm 1.3) and remained high until the end of the study. Group 6, vaccinated with pcDNA3.1 constructs encoding the two non-structural proteins μ NS and σ NS and the cellular attachment protein σ 1, showed lower viral RNA loads in the blood samples throughout the challenge, and at 8 wpc, the mean PRV Ct-value (25.1 (\pm 5.4)) was significantly lower ($p = 0.002$) than in the control group (16.6 (\pm 1.5)) (Figure 3B). The viral kinetics for this group was also delayed compared to the control group, and PRV levels did not peak in blood until the end of the challenge (10 wpc, Ct-value of 20.1 (\pm 4.5)). In contrast, the viral loads in blood from all five groups vaccinated with pSAV replicon-based constructs were overall high. However, even though not significant, pSAV expressing μ NS and σ NS (Group 2) had lower viral RNA levels than the control group at the end of the study (10 wpc), with a mean Ct-value of 18.8 (\pm 3.9). pSAV replicons encoding all ten PRV proteins (pSAV/ μ NS + μ 1 + μ 2 + σ NS + σ 1 + σ 2 + σ 3 + λ 1 + λ 2 + λ 3; Group 5) showed delayed PRV kinetics with a peak load of viral RNA at 10 wpc (Ct-value of 16.4 (\pm 2.5), Figure 3A). At 6 wpc, Groups 2, 3 and 5 all had significantly lower viral RNA load (p -values of 0.048, 0.023 and 0.026, respectively), than the control group.

pcDNA3.1 expressing μ NS + σ NS + σ 1 significantly reduced HSMI histopathological lesions

Histopathological lesions in the heart typical for HSMI were present in the pSAV EGFP control group at 6 wpc, peaked at 8 wpc with a mean score of 2.7 and 3.3 in the epicardium and ventricle respectively. The lesions gradually resolved towards 10 wpc. The heart lesions were characterized by massive epicarditis and infiltration of lymphocytic cells in the compact and spongy myocardium layer of the ventricle. Mean histopathological scores from all vaccination groups and the control group from vaccination trial #I are showed in Figure 5. All vaccinated

groups had lower heart pathology scores compared to the control group at its peaking point. Group 6, pcDNA3.1 expressing $\mu\text{NS} + \sigma\text{NS} + \sigma 1$, had reduced heart pathology in both the epicardium and the ventricle at all sampling points post challenge and absence of heart lesions at 8 wpc (score of 0.0) ($p = 0.002$) in both compartments, at the time when the pSAV/EGFP control group peaked (Figure 4). At 10 wpc, 2 out of 6 fish in Group 6 showed histopathological changes with a mean score for the group of 0.5 for both epicardium and ventricle. Group 1 (pSAV/ μNS) and Group 2 (pSAV/ $\mu\text{NS} + \sigma\text{NS}$) both peaked in heart pathology 8 wpc, like the control group, while Groups 3, 4 and 5 (pSAV/ $\mu\text{NS} +$ various structural proteins) peaked at 6 wpc.

Vaccination trial #II

pcDNA3.1 vaccine expressing $\mu\text{NS} + \sigma\text{NS} + \sigma 1$ reduced viral load in blood

In vaccine trial II, RT-qPCR analysis indicated high PRV loads at the two sampling points 6 and 8 wpc for both control groups with peak viral load at 6 wpc for the pcDNA3.1/EGFP group (mean Ct-value of 16.1 ± 3.3) and at 8 wpc for the PBS group (mean Ct-value of 17.1 ± 0.9). All four vaccinated groups showed reduced viral RNA loads in blood cells compared to the controls at 6 wpc (Figure S2A). Group 1 (pcDNA3.1/ $\mu\text{NS} + \sigma\text{NS} + \sigma 1$) showed significantly lower viral RNA load 6 wpc with a mean Ct-value of $24.2 (\pm 5.3)$ ($p = 0.012$ and $p = 0.035$ compared to the pcDNA3.1/EGFP and the PBS groups, respectively), before peaking with a Ct-value of $18.7 (\pm 1.8)$ at 8 wpc (Figure 6). Group 4 (pcDNA3.1/ μNS) was the only group that showed higher PRV RNA levels than the control groups at 8 wpc, with a mean Ct-value of $15.6 (\pm 2.0)$.

pcDNA3.1 vaccine expressing $\mu\text{NS} + \sigma\text{NS} + \sigma 1$ reduced viral loads in plasma

In general, the pattern of viral RNA levels in plasma from vaccination trial II was similar to that of the viral RNA levels in blood, for all vaccination groups (Figure S2B). The pSAV/EGFP control group peaked at 8 wpc with a mean Ct-value of $26.0 (\pm 1.2)$ and the PBS group peaked at 6 wpc with a mean Ct-value of $26.7 (\pm 6.4)$. Group 1 (pcDNA3.1/ $\mu\text{NS} + \sigma\text{NS} + \sigma 1$) showed significantly reduced PRV RNA levels in plasma at both 6 and 8 wpc with a mean Ct-value of $32.8 (\pm 3.5)$ at 6 wpc ($p = 0.000$ and $p = 0.011$ compared to the pcDNA3.1/EGFP group and the PBS group), and $28.0 (\pm 2.5)$ at 8 wpc ($p = 0.021$ and $p = 0.013$ compared to the pcDNA3.1/EGFP group and the PBS group, respectively) (Figure 6). At 6 wpc, Group 2 (pcDNA3.1/ $\mu\text{NS} + \sigma\text{NS} + \sigma 3$) and Group 3 (pcDNA3.1/ $\mu\text{NS} + \sigma\text{NS}$), also showed significantly reduced viral RNA levels in plasma with Ct-values of $30.0 (\pm 4.8)$ and $31.1 (\pm 4.3)$, compared

to the pSAV/EGFP ($p= 0.014$ and 0.007 , respectively) and the PBS ($p = 0.023$ and 0.013 , respectively) control groups. Group 4 (pcDNA3.1/ μ NS) showed higher PRV load than the control groups 6 wpc, with a mean Ct-value of $27.2 (\pm 5.3)$.

pcDNA3.1 vaccine expressing μ NS + σ NS + σ 1 reduced histopathological lesions in heart

Histopathological lesions in the heart typical for HSMI were present in all fish from the control groups at 8 wpc, with a mean histopathological score of $2.5 (\pm 0.2)$ and $4.0 (\pm 0.0)$ in the epicardium and the ventricle, respectively, for the pSAV/EGFP group, and $2.1 (\pm 0.4)$ and $4.0 (\pm 0.0)$ for the PBS group. Mean histopathological scores for all groups from vaccination trial #II are shown in Figure 7. Group 1 (pcDNA3.1/ μ NS + σ NS + σ 1) showed reduced histopathological lesions 8 wpc with mean score of $1.0 (\pm 0.6)$ and $2.5 (\pm 1.5)$ in the epicardium and ventricle, respectively (Figure 8). Group 2, 3 and 4 also showed reduced histopathological scores in both epicardium and ventricle at 8 wpc, although the results are not significant compared to the control groups.

Discussion

In this study, a pcDNA3.1-based vaccine expressing the PRV non-structural protein μ NS, the non-structural protein σ NS, and the cell attachment protein σ 1 [12], delayed the kinetics of PRV infection and induced protection against HSMI in Atlantic salmon. The vaccine was tested in two independent experimental infection trials, inducing full protection against HSMI (as determined by histopathology) in the first trial and moderate protection in the second trial. Various combinations of DNA-layered alphavirus-replicon constructs were tested in Trial #I, where PRV μ NS was expressed alone or in combination with σ NS and structural PRV capsid and core proteins. All vaccine constructs reduced HSMI-specific heart lesions compared with the control group in this trial. However, the group vaccinated by the pcDNA3.1/ μ NS + σ NS + σ 1 combination was better protected than any of the groups vaccinated by replicon constructs. Consequently, in Trial #II, various constructs using the pcDNA3.1 backbone were compared.

PRV protein expression from pSAV replicon constructs and pcDNA3.1 was confirmed *in vitro* in Chinook salmon embryo (CHSE) before used *in vivo* in the vaccination trial. [40]. As visualized by fluorescent microscopy, more cells seemed transfected by the pcDNA3.1 constructs than the pSAV replicon constructs. The larger pSAV replicon backbone (12 073 bp) compared to the pcDNA3.1 backbone (5434 bp) could partly explain the differences in

transfection efficiency; transfection with larger DNA constructs are generally less efficient [42]. The amount of PRV protein expressed in the transfected cells increased from 24 h until 96 h post transfection regardless of the expression vector. This is consistent with earlier studies, which have shown that expression from pSAV replicon constructs peak at day 4 post transfection [40, 43]. Previous studies have shown that expression of EGFP from pSAV/EGFP transfected CHSE-214 cells is delayed and lower compared to the reporter expressed under the control of an immediate-early CMV promoter, which is the promoter in pcDNA3.1 [43]. This is in line with the results from the transfections in this study. The delay in protein production from pSAV constructs could be explained by the pSAV expression mechanisms, which require transcription and translation of the alphaviral replicase complex and transcription of the copy (minus) strand of the genome before transcription of the subgenomic ORF containing the PRV coding sequences. The higher efficiency of protein expression from pcDNA3.1 motivated us to test them in the second trial.

Control groups with mock-vaccinated fish were surveilled to verify proper infection after experimental challenge with PRV. In challenge Trial #I, the control group was vaccinated with pSAV/EGFP, whereas in challenge Trial #II, two control groups were used; one vaccinated with a pcDNA3.1/EGFP construct and one given PBS. PRV RNA levels were analyzed in blood (Trial #I and II) and plasma (Trial #II), reaching peak levels approximately 6 wpc in control groups, which is in line with previous cohabitant PRV challenge experiments [15]. In addition, typical histopathological lesions were observed in the heart and thus the criteria for the diagnosis of HSMI were fulfilled [8, 44]. Ideally, vaccine candidates should also be tested for protection against mortality compared to mock-vaccinated or unvaccinated fish groups, however, mortality has not been observed in PRV challenge experiments [7, 11, 14].

All five vaccine combinations of the pSAV-replicon constructs contained the gene for the non-structural μ NS protein, which is responsible for organization of viral factories during PRV replication [21]. The PRV μ NS protein forms dense, globular, cytoplasmic inclusions and recruits other PRV proteins to a workshop for virus replication and assembly [21]. One of the pSAV based vaccines contained only the μ NS-expressing construct, and the typical μ NS inclusions were observed when the construct was transfected into CHSE-214 cells. This vaccine altered the kinetics of PRV infection, but protection against HSMI was only moderate, suggesting that μ NS does not induce a sufficient protective immune response. The second pSAV replicon construct combination expressed μ NS and σ NS, which is another non-structural

protein linked to viral factories. The functional properties of the σ NS protein has not been studied for PRV, but for MRV σ NS has been shown to facilitate the assembly of virus particles [45, 46]. Here, adding the σ NS construct to the vaccine did not improve protection. In the third and fourth vaccine combinations, the μ NS and σ NS constructs were kept, while the core proteins; λ 1, λ 3, μ 2 and σ 2, and the outer capsid proteins; λ 2, μ 1, σ 1 and σ 3 were added, respectively. Neither of these vaccines reduced the PRV RNA levels in blood compared to the control, but a moderate reduction in cardiac histopathological scores was again observed. In the fifth DNA-layered pSAV-replicon vaccine group, all the primary ORFs of the PRV genomic segments were expressed, and although the viral RNA levels were not significantly reduced compared to the control group at 8 wpc, the histopathological score was significantly lower than that of the control group at the time of maximum change, i.e. 8 wpc. In conclusion, the various combinations of the pSAV replicon construct expressing non-structural and structural PRV proteins did only induce moderate protection against HSMI. Alphavirus replicons have previously been demonstrated to express dsRNA and to induce stronger T cell immune response than conventional DNA vectors [43, 47]. The pathogenesis of HSMI is not fully elucidated, but the histopathological lesions are characterized by influx of inflammatory cells, indicating that immune-mediated mechanisms are responsible for development of the disease [16].

In the first vaccine trial, the combination μ NS, σ NS and σ 1 expressed by pcDNA3.1 vectors, reduced the virus load significantly and almost completely abolished histopathological changes. Therefore, the second experimental challenge was set up with pcDNA3.1 expression vectors only. Two of the vaccination groups were similar to the earlier groups, only changing from pSAV replicon vectors to pcDNA3.1 expression vectors. However, in the second experimental trial, although viral RNA levels and heart pathological lesions were reduced compared to the control groups, the protection was only moderate. The two experimental challenges were set up with similar experimental conditions. Environmental factors however, cannot be completely controlled and might have affected the different outcomes. Fish with different genetic backgrounds were used in the two trials. However, HSMI has been detected in all salmon producing counties in Norway [48], and there are currently no indications that the virus shows selective preference for specific salmon strains or families. Control fish in both experimental challenges showed similar infection kinetics following the cohabitant infection confirming equal infection efficiency in both salmon groups. Also, even though more fish were

sampled in the second experimental challenge, they were only sampled twice, which might have led to information loss regarding viral kinetics.

In Trial #I, the combination μ NS, σ NS and σ 1, which induced full protection when expressed through the pcDNA3.1 backbones, was also included in the pSAV replicons groups, in the vaccines that contained the outer capsid proteins (Groups 4 and 5). Interestingly, this combination only induced minor protection suggesting that the amount of expressed protein might be more important for protection against HSMI than the stronger immune responses induced by alphavirus replicons [41, 47]. In these experimental trials, PRV was transmitted by cohabitation and thus needed to cross the mucosal barrier in order to establish an infection. IgT is the specific mucosal antibody in salmonids [49]. The differences in efficacy between the pSAV and pcDNA3.1 constructs could be that the induction of protection at mucosal sites or of mechanisms blocking PRV dissemination to erythrocytes are dependent upon viral protein expression levels.

Besides the μ NS, σ NS and σ 1 combination, three other pcDNA3.1 based vaccines were tested in vaccination trial #II. In Group 2, the μ NS and σ NS proteins were expressed in combination with the outer capsid protein σ 3. The σ 3 protein is a major part of the outer virion surface forming a heterohexamer with μ 1 [50]. Compared to the control groups, viral RNA load in plasma was significantly reduced at 6 wpc and partly reduced in both blood and plasma at 8 wpc. In the third pcDNA3.1 vaccine group, μ NS was expressed in combination with σ NS only. Here, the viral RNA load in plasma was significantly reduced at both sampling points compared to the control groups. Both Group 2 and 3 also showed reduced histopathological lesions in the heart at the end of the challenge, although the reduction was not significant. The last pcDNA3.1 vaccine group expressed μ NS alone, and in this group, viral RNA load was similar or even increased compared to the control groups. However, this vaccine also offered reduced histopathological lesions in the heart at 8 wpc, indicating that the viral factory forming protein could be a suitable target for immunization even though μ NS alone was not capable of mounting a desired response.

A number of combinations of PRV proteins expressed as DNA vaccines was tested in the present study. Co-expression of μ NS, σ NS and σ 1, combining two non-structural proteins and the putative receptor-binding protein, proved advantageous. The intracellular non-structural proteins are most likely to trigger a cell-mediated immune response while the outer capsid

protein $\sigma 1$ is a good candidate for the production of neutralizing antibodies. In addition, abundant expression from the pcDNA3.1 vector might have been decisive for the recruitment and activation of the immune system. The mechanism of protection after successful vaccination against viral infection in fish is not fully understood and might depend upon several factors. Studies on both IPNV and PD have claimed good correlation between antibody titer or neutralizing antibodies and protection acquired after vaccination trials, indicating that the humoral immune response might be important for protection [51, 52]. A study on ISAV showed a stronger correlation between survival and the cell mediated immune response [53]. Based on this, it is likely that a combined response provides the most optimal protection. Antibodies that interfere with cell attachment, endosomal release or viral uncoating, i.e antibodies against $\sigma 1$, $\sigma 3$, $\mu 1c$, and $\lambda 2$, have been demonstrated to inhibit MRV infection in both cell culture systems and mice [32, 54, 55]. It is thus likely that σ and μ protein analogues in PRV are targets for antibodies in infected salmon and that neutralizing antibodies may have a role in defense against the virus [56]. A recent report using novel bead-based assays for detection of specific antibodies against PRV proteins, demonstrated a distinct increase in specific antibodies against $\mu 1C$ and μNS in plasma of Atlantic salmon during the course of an experimental PRV challenge [56]. The mechanisms of protection in the current vaccination trials were not elucidated and future studies should include antibody assays as well as gene expression analysis focusing on the immune response.

Aquaculture confine animals under high density which generally facilitate transmission of infectious agents and reduced resistance to disease [57], and vaccination to control infectious diseases is necessary for the sustainability of aquaculture. PRV has been detected in various salmonid species; Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*), chum salmon (*O. keta*), cutthroat trout (*O. clarkii*) and brown trout (*Salmo trutta*) [48, 58] and PRV's ability to infect several species and having a segmented genome prone to re-assortment, are factors that may ease rapid evolution. Controlling PRV infection may therefore limit a considerable risk factor for the aquaculture industry, and the development of protective vaccine candidates against HSMI is an important step. With the estimated future growth in aquaculture, proper disease control depends upon development of efficient vaccines.

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References

1. Wessel O, Braaen S, Alarcon M, Haatveit H, Roos N, Markussen T, Tengs T, Dahle MK, Rimstad E: **Infection with purified Piscine orthoreovirus demonstrates a causal relationship with heart and skeletal muscle inflammation in Atlantic salmon.** *PloS One* 2017, **12**(8):e0183781.
2. Norwegian veterinary institute: **Fish health report 2016.** Edited by Hjeltnes H, Walde C, Bang Jensen B, Haukaas A. Oslo; 2017.
3. Ferguson HW, Kongtorp RT, Taksdal T, Graham D, Falk K: **An outbreak of disease resembling heart and skeletal muscle inflammation in Scottish farmed salmon, *Salmo salar* L., with observations on myocardial regeneration.** *J Fish Dis* 2005, **28**(2):119-123.
4. Godoy MG, Kibenge MJ, Wang Y, Suarez R, Leiva C, Vallejos F, Kibenge FS: **First description of clinical presentation of piscine orthoreovirus (PRV) infections in salmonid aquaculture in Chile and identification of a second genotype (Genotype II) of PRV.** *Virol J* 2016, **13**(1):98.
5. Di Cicco E, Ferguson HW, Schulze AD, Kaukinen KH, Li S, Vanderstichel R, Wessel O, Rimstad E, Gardner IA, Hammell KL *et al*: **Heart and skeletal muscle inflammation (HSMI) disease diagnosed on a British Columbia salmon farm through a longitudinal farm study.** *PloS One* 2017, **12**(2):e0171471.
6. Kongtorp RT, Halse M, Taksdal T, Falk K: **Longitudinal study of a natural outbreak of heart and skeletal muscle inflammation in Atlantic salmon, *Salmo salar* L.** *J Fish Dis* 2006, **29**(4):233-244.
7. Kongtorp RT, Kjerstad A, Taksdal T, Guttvik A, Falk K: **Heart and skeletal muscle inflammation in Atlantic salmon, *Salmo salar* L: a new infectious disease.** *J Fish Dis* 2004, **27**.
8. Kongtorp RT, Taksdal T, Lyngoy A: **Pathology of heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon *Salmo salar*.** *Dis Aquatic Org* 2004, **59**(3):217-224.

9. Takano T, Nawata A, Sakai T, Matsuyama T, Ito T, Kurita J, Terashima S, Yasuike M, Nakamura Y, Fujiwara A *et al*: **Full-Genome sequencing and confirmation of the causative agent of Erythrocytic inclusion body syndrome in Coho Salmon identifies a new type of Piscine Orthoreovirus.** *PloS One* 2016, **11**(10):e0165424.
10. Olsen AB, Hjortaas M, Tengs T, Hellberg H, Johansen R: **First description of a new disease in Rainbow Trout (*Oncorhynchus mykiss* (Walbaum)) similar to Heart and skeletal muscle inflammation (HSMI) and detection of a gene sequence related to Piscine Orthoreovirus (PRV).** *PloS One* 2015, **10**(7):e0131638.
11. Palacios G, Lovoll M, Tengs T, Hornig M, Hutchison S, Hui J, Kongtorp RT, Savji N, Bussetti AV, Solovyov A *et al*: **Heart and skeletal muscle inflammation of farmed salmon is associated with infection with a novel reovirus.** *PloS One* 2010, **5**(7):e11487.
12. Markussen T, Dahle MK, Tengs T, Løvoll M, Finstad Ø, Wiik-Nielsen CR, Grove S, Lauksund S, Robertsen B, Rimstad E: **Sequence analysis of the genome of piscine orthoreovirus (PRV) associated with heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (*Salmo salar*).** *PloS One* 2013, **8**.
13. Palacios G, Løvoll M, Tengs T, Hornig M, Hutchison S, Hui J, Kongtorp RT, Savji N, Bussetti AV, Solovyov A *et al*: **Heart and skeletal muscle inflammation of farmed salmon is associated with infection with a novel reovirus.** *PloS One* 2010, **5**.
14. Finstad OW, Dahle MK, Lindholm TH, Nyman IB, Lovoll M, Wallace C, Olsen CM, Storset AK, Rimstad E: **Piscine orthoreovirus (PRV) infects Atlantic salmon erythrocytes.** *Vet Res* 2014, **45**:35.
15. Finstad OW, Falk K, Lovoll M, Evensen O, Rimstad E: **Immunohistochemical detection of piscine reovirus (PRV) in hearts of Atlantic salmon coincide with the course of heart and skeletal muscle inflammation (HSMI).** *Vet Res* 2012, **43**:27.
16. Haatveit HM, Wessel O, Markussen T, Lund M, Thiede B, Nyman IB, Braaen S, Dahle MK, Rimstad E: **Viral Protein Kinetics of Piscine Orthoreovirus Infection in Atlantic Salmon Blood Cells.** *Viruses* 2017, **9**(3).

17. Wessel O, Olsen CM, Rimstad E, Dahle MK: **Piscine orthoreovirus (PRV) replicates in Atlantic salmon (*Salmo Salar L.*) erythrocytes ex vivo.** *Vet Res* 2015, **In press**.
18. de Castro IF, Volonte L, Risco C: **Virus factories: biogenesis and structural design.** *Cell microbiol* 2013, **15(1):24-34**.
19. Carroll K, Hastings C, Miller CL: **Amino acids 78 and 79 of Mammalian Orthoreovirus protein microNS are necessary for stress granule localization, core protein lambda 2 interaction, and de novo virus replication.** *Virology* 2014, **448:133-145**.
20. Miller CL, Arnold MM, Broering TJ, Hastings CE, Nibert ML: **Localization of mammalian orthoreovirus proteins to cytoplasmic factory-like structures via nonoverlapping regions of microNS.** *J Virol* 2010, **84(2):867-882**.
21. Haatveit HM, Nyman IB, Markussen T, Wessel O, Dahle MK, Rimstad E: **The non-structural protein muNS of piscine orthoreovirus (PRV) forms viral factory-like structures.** *Vet Res* 2016, **47(1):5**.
22. Dermody TS, Nibert ML, Bassel-Duby R, Fields BN: **A sigma 1 region important for hemagglutination by serotype 3 reovirus strains.** *J Virol* 1990, **64(10):5173-5176**.
23. Kirchner E, Guglielmi KM, Strauss HM, Dermody TS, Stehle T: **Structure of reovirus sigma1 in complex with its receptor junctional adhesion molecule-A.** *PLoS Path* 2008, **4(12):e1000235**.
24. Lee PW, Hayes EC, Joklik WK: **Protein sigma 1 is the reovirus cell attachment protein.** *Virology* 1981, **108(1):156-163**.
25. Barton ES, Forrest JC, Connolly JL, Chappell JD, Liu Y, Schnell FJ, Nusrat A, Parkos CA, Dermody TS: **Junction adhesion molecule is a receptor for reovirus.** *Cell* 2001, **104(3):441-451**.
26. Reiter DM, Frierson JM, Halvorson EE, Kobayashi T, Dermody TS, Stehle T: **Crystal structure of reovirus attachment protein sigma1 in complex with sialylated oligosaccharides.** *PLoS Path* 2011, **7(8):e1002166**.

27. Weiner HL, Fields BN: **Neutralization of reovirus: the gene responsible for the neutralization antigen.** *J Exp Med* 1977, **146**(5):1305-1310.
28. Dryden KA, Wang G, Yeager M, Nibert ML, Coombs KM, Furlong DB, Fields BN, Baker TS: **Early steps in reovirus infection are associated with dramatic changes in supramolecular structure and protein conformation: analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction.** *J Cell Biol* 1993, **122**(5):1023-1041.
29. Nibert ML, Furlong DB, Fields BN: **Mechanisms of viral pathogenesis. Distinct forms of reoviruses and their roles during replication in cells and host.** *J Clin Invest* 1991, **88**(3):727-734.
30. Nibert ML, Fields BN: **A carboxy-terminal fragment of protein mu 1/mu 1C is present in infectious subvirion particles of mammalian reoviruses and is proposed to have a role in penetration.** *J Virol* 1992, **66**(11):6408-6418.
31. Skehel JJ, Joklik WK: **Studies on the in vitro transcription of reovirus RNA catalyzed by reovirus cores.** *Virology* 1969, **39**(4):822-831.
32. Tyler KL, Mann MA, Fields BN, Virgin HW: **Protective anti-reovirus monoclonal antibodies and their effects on viral pathogenesis.** *J virol* 1993, **67**(6):3446-3453.
33. Wolf A, Hodneland K, Frost P, Braaen S, Rimstad E: **A hemagglutinin-esterase-expressing salmonid alphavirus replicon protects Atlantic salmon (*Salmo salar*) against infectious salmon anemia (ISA).** *Vaccine* 2013, **31**(4):661-669.
34. Hikke MC, Braaen S, Villoing S, Hodneland K, Geertsema C, Verhagen L, Frost P, Vlak JM, Rimstad E, Pijlman GP: **Salmonid alphavirus glycoprotein E2 requires low temperature and E1 for virion formation and induction of protective immunity.** *Vaccine* 2014, **32**(47):6206-6212.
35. Lorenzen N, Lorenzen E, Einer-Jensen K, Heppell J, Davis HL: **Genetic vaccination of rainbow trout against viral haemorrhagic septicaemia virus: small amounts of plasmid DNA protect against a heterologous serotype.** *Virus Res* 1999, **63**(1-2):19-25.

36. Salenius K, Simard N, Harland R, Ulmer JB: **The road to licensure of a DNA vaccine.** *Curr Opin Investig Drugs* 2007, **8**(8):635-641.
37. Rayner JO, Dryga SA, Kamrud KI: **Alphavirus vectors and vaccination.** *Rev Med Virol* 2002, **12**(5):279-296.
38. Karlsen M, Villoing S, Rimstad E, Nylund A: **Characterization of untranslated regions of the salmonid alphavirus 3 (SAV3) genome and construction of a SAV3 based replicon.** *Virol J* 2009, **6**:173.
39. Karlsen M, Villoing S, Ottem KF, Rimstad E, Nylund A: **Development of infectious cDNA clones of Salmonid alphavirus subtype 3.** *BMC Res Not* 2010, **3**:241.
40. Abdullah A, Olsen CM, Hodneland K, Rimstad E: **A polyprotein-expressing salmonid alphavirus replicon induces modest protection in atlantic salmon (*Salmo salar*) against infectious pancreatic necrosis.** *Viruses* 2015, **7**(1):252-267.
41. Leitner WW, Hwang LN, deVeer MJ, Zhou A, Silverman RH, Williams BR, Dubensky TW, Ying H, Restifo NP: **Alphavirus-based DNA vaccine breaks immunological tolerance by activating innate antiviral pathways.** *Nat Med* 2003, **9**(1):33-39.
42. Hornstein BD, Roman D, Arevalo-Soliz LM, Engevik MA, Zechiedrich L: **Effects of Circular DNA Length on Transfection Efficiency by Electroporation into HeLa Cells.** *PloS One* 2016, **11**(12):e0167537.
43. Olsen CM, Pemula AK, Braaen S, Sankaran K, Rimstad E: **Salmonid alphavirus replicon is functional in fish, mammalian and insect cells and in vivo in shrimps (*Litopenaeus vannamei*).** *Vaccine* 2013, **31**(48):5672-5679.
44. Mikalsen AB, Haugland O, Rode M, Solbakk IT, Evensen O: **Atlantic salmon reovirus infection causes a CD8 T cell myocarditis in Atlantic salmon (*Salmo salar* L.).** *PloS One* 2012, **7**(6):e37269.
45. Becker MM, Peters TR, Dermody TS: **Reovirus sigma NS and mu NS proteins form cytoplasmic inclusion structures in the absence of viral infection.** *J Virol* 2003, **77**(10):5948-5963.

46. Broering TJ, Kim J, Miller CL, Piggott CD, Dinoso JB, Nibert ML, Parker JS: **Reovirus nonstructural protein muNS recruits viral core surface proteins and entering core particles to factory-like inclusions.** *J Virol* 2004, **78**(4):1882-1892.
47. Knudsen ML, Mbewe-Mvula A, Rosario M, Johansson DX, Kakoulidou M, Bridgeman A, Reyes-Sandoval A, Nicosia A, Ljungberg K, Hanke T *et al*: **Superior induction of T cell responses to conserved HIV-1 regions by electroporated alphavirus replicon DNA compared to that with conventional plasmid DNA vaccine.** *J virol* 2012, **86**(8):4082-4090.
48. Garseth Å, Fritsvold C, Opheim M, Skjerve E, Biering E: **Piscine reovirus (PRV) in wild Atlantic salmon, *Salmo salar* L., and sea-trout, *Salmo trutta* L., in Norway.** *J Fish Dis* 2013, **36**.
49. Zhang YA, Salinas I, Li J, Parra D, Bjork S, Xu Z, LaPatra SE, Bartholomew J, Sunyer JO: **IgT, a primitive immunoglobulin class specialized in mucosal immunity.** *Nat Immunol* 2010, **11**(9):827-835.
50. Liemann S, Chandran K, Baker TS, Nibert ML, Harrison SC: **Structure of the reovirus membrane-penetration protein, Mu1, in a complex with its protector protein, Sigma3.** *Cell* 2002, **108**(2):283-295.
51. Munang'andu HM, Fredriksen BN, Mutoloki S, Dalmo RA, Evensen Ø: **Antigen dose and humoral immune response correspond with protection for inactivated infectious pancreatic necrosis virus vaccines in Atlantic salmon (*Salmo salar* L).** *Vet Res* 2013, **44**(1):7.
52. Graham DA, Rowley HR, Frost P: **Cross-neutralization studies with salmonid alphavirus subtype 1-6 strains: results with sera from experimental studies and natural infections.** *J Fish Dis* 2014, **37**(8):683-691.
53. Jorgensen SM, Afanasyev S, Krasnov A: **Gene expression analyses in Atlantic salmon challenged with infectious salmon anemia virus reveal differences between individuals with early, intermediate and late mortality.** *BMC genomics* 2008, **9**:179.
54. Helander A, Miller CL, Myers KS, Neutra MR, Nibert ML: **Protective immunoglobulin A and G antibodies bind to overlapping intersubunit epitopes in**

- the head domain of type 1 reovirus adhesin sigma1.** *J Virol* 2004, **78**(19):10695-10705.
55. Hutchings AB, Helander A, Silvey KJ, Chandran K, Lucas WT, Nibert ML, Neutra MR: **Secretory immunoglobulin A antibodies against the sigma1 outer capsid protein of reovirus type 1 Lang prevent infection of mouse Peyer's patches.** *J Virol* 2004, **78**(2):947-957.
56. Teige LH, Lund M, Haatveit HM, Rosaeg MV, Wessel O, Dahle MK, Storset AK: **A bead based multiplex immunoassay detects Piscine orthoreovirus specific antibodies in Atlantic salmon (*Salmo salar*).** *Fish Shellfish Immunol* 2017, **63**:491-499.
57. Rimstad E: **Examples of emerging virus diseases in salmonid aquaculture.** *Aquacult Res* 2011, **42**:86-89.
58. Kibenge MJ, Iwamoto T, Wang Y, Morton A, Godoy MG, Kibenge FS: **Whole-genome analysis of piscine reovirus (PRV) shows PRV represents a new genus in family Reoviridae and its genome segment S1 sequences group it into two separate sub-genotypes.** *Virol J* 2013, **10**:230.

Tables

Table 1. Vaccine groups

The combination of vaccine antigens and the expression vector backbone in Trial #I and Trial #II.

Vaccination trial #I		
Group	Vector	Vaccine
1	pSAV	μ NS
2	pSAV	μ NS + σ NS
3	pSAV	μ NS + μ 2 + σ NS + σ 2 + λ 1 + λ 3
4	pSAV	μ NS + μ 1 + σ NS + σ 1 + σ 3 + λ 2
5	pSAV	μ NS + μ 1 + μ 2 + σ NS + σ 1 + σ 2 + σ 3 + λ 1 + λ 2 + λ 3
6	pcDNA3.1	μ NS + σ NS + σ 1
7	pSAV	EGFP (control)

Vaccination trial #II		
Group	Vector	Vaccine
1	pcDNA3.1	μ NS + σ NS + σ 1
2	pcDNA3.1	μ NS + σ NS + σ 3
3	pcDNA3.1	μ NS + σ NS
4	pcDNA3.1	μ NS
5	pcDNA3.1	EGFP (control)
6	pcDNA3.1	PBS (control)

Figures

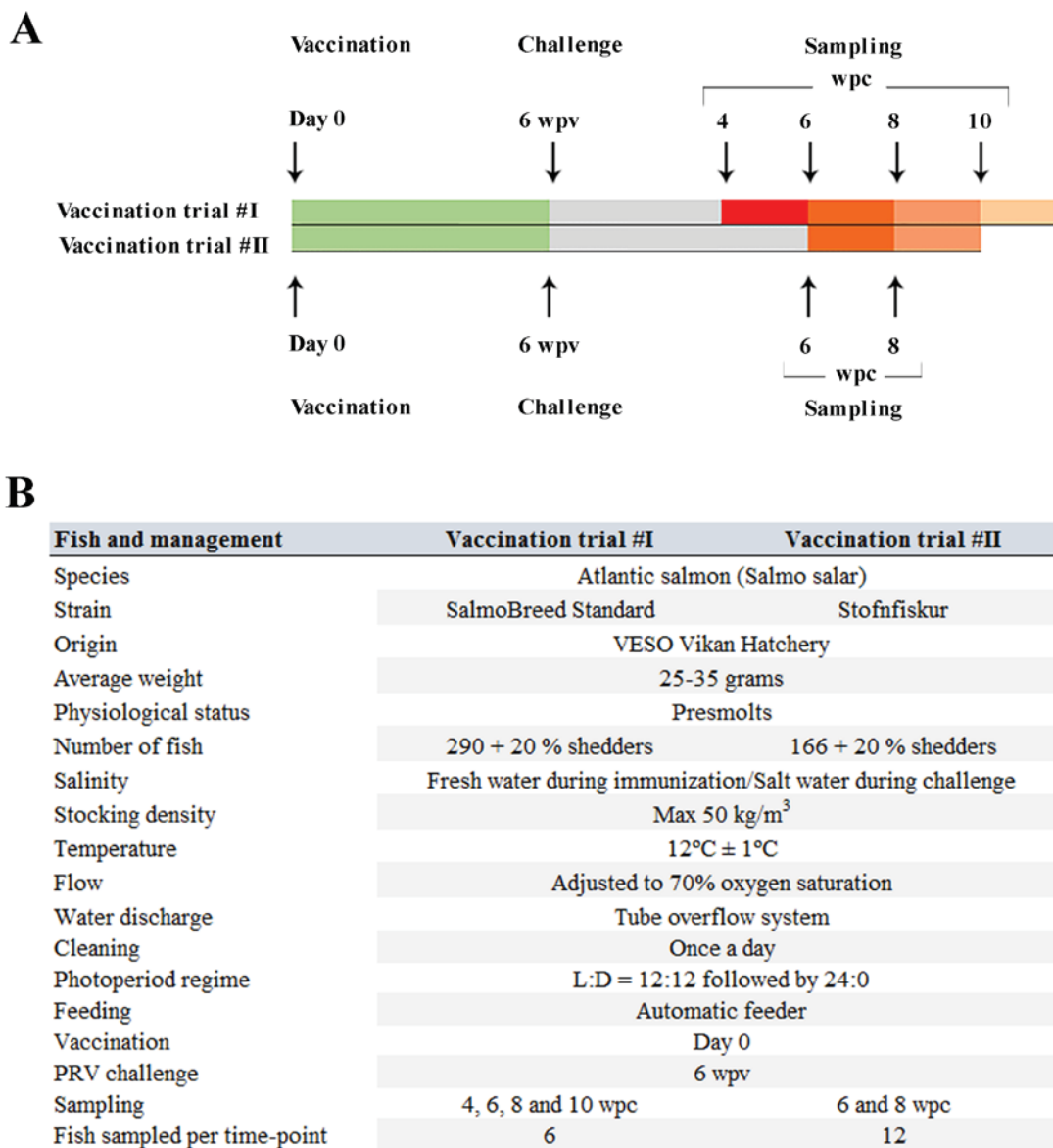


Figure 1. Vaccine trial setup

(A) The time-course and sampling points for the two vaccination trials. (B) Table containing the experimental conditions of the two experimental trials. wpv = weeks post vaccination. wpc = weeks post challenge.

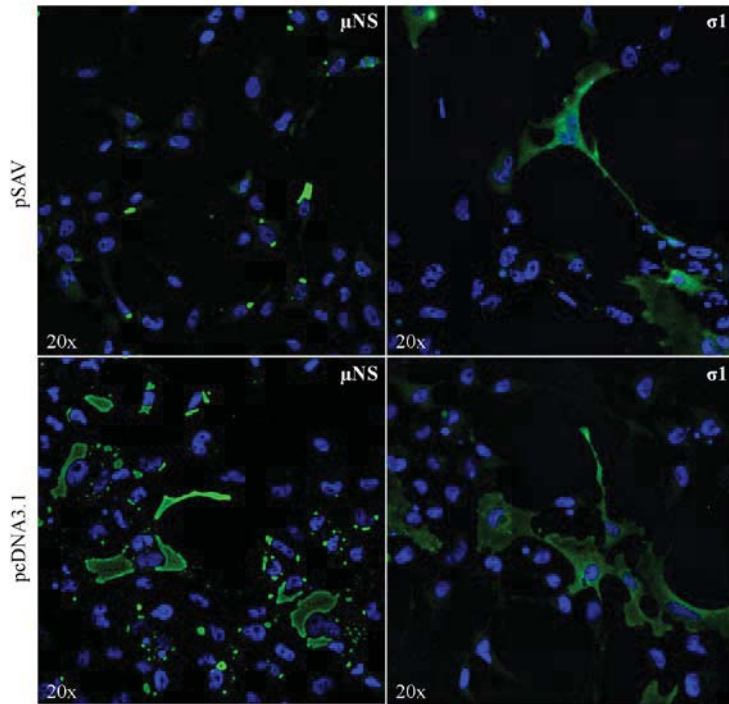


Figure 2. Expression of μ NS and σ 1 from vaccine vectors in fish cells

CHSE cells expressing the non-structural protein μ NS and the structural protein σ 1 after transfection with pSAV replicon constructs (top row) or pcDNA3.1 constructs (bottom row). The cells were processed for fluorescence microscopy 96 h post transfection and the nuclei were stained with Hoechst (blue).

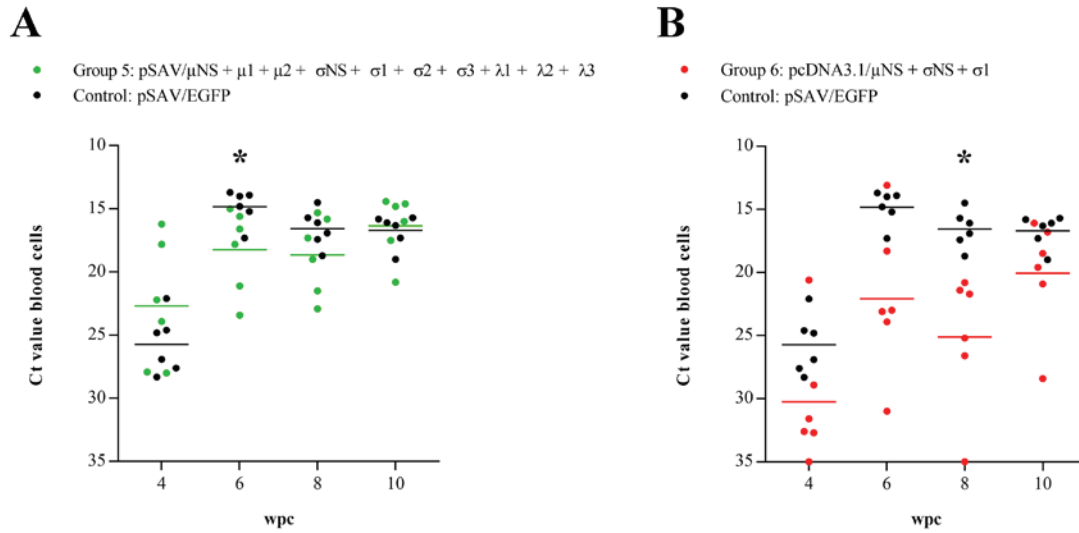


Figure 3. Vaccine trial #I: Piscine orthoreovirus (PRV) RNA load in blood cells

RT-qPCR of PRV gene segment S1 in blood cells from cohabitant fish in vaccination trial #I. (A) Group 5 (pSAV/μNS + μ1 + μ2 + σNS + σ1 + σ2 + σ3 + λ1 + λ2 + λ3) and pSAV/EGFP control. (B) Group 6 (pcDNA3.1/μNS + σNS + σ1) and pSAV/EGFP control. Ct-values of individual fish (dots) and mean (line). The Ct-values from all individuals within the separate groups are compared to the individual Ct-values from the control group at each timepoint using the Mann Whitney compare ranks test; Star = p - value \leq 0.05. n = 6 per time-point. wpc = weeks post challenge.

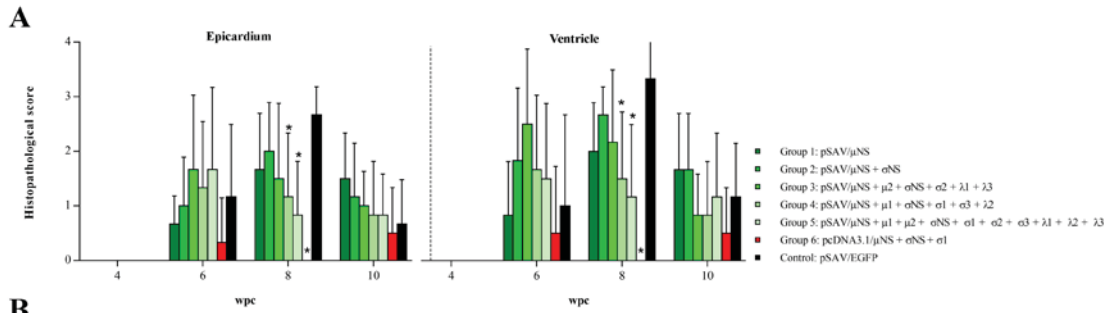


Figure 5. Vaccine trial #I: Histopathological scores in the epicardium and ventricle

(A) Bars illustrating mean histopathological score with SD in epicardium and ventricle from each timepoint of sampling. (B) Table showing mean histopathological score in epicardium and ventricle from each group at each timepoint of sampling. The histopathological scores from all individuals within the separate groups are compared to the individual histopathological scores from the control group at each timepoint using the Mann Whitney compare ranks test; Star = p - value ≤ 0.05 . n = 6. wpc = weeks post challenge.

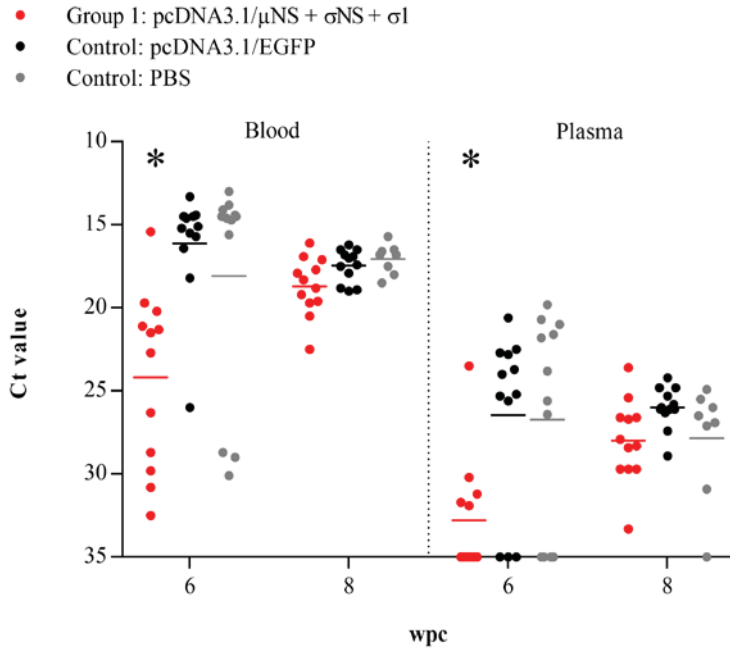
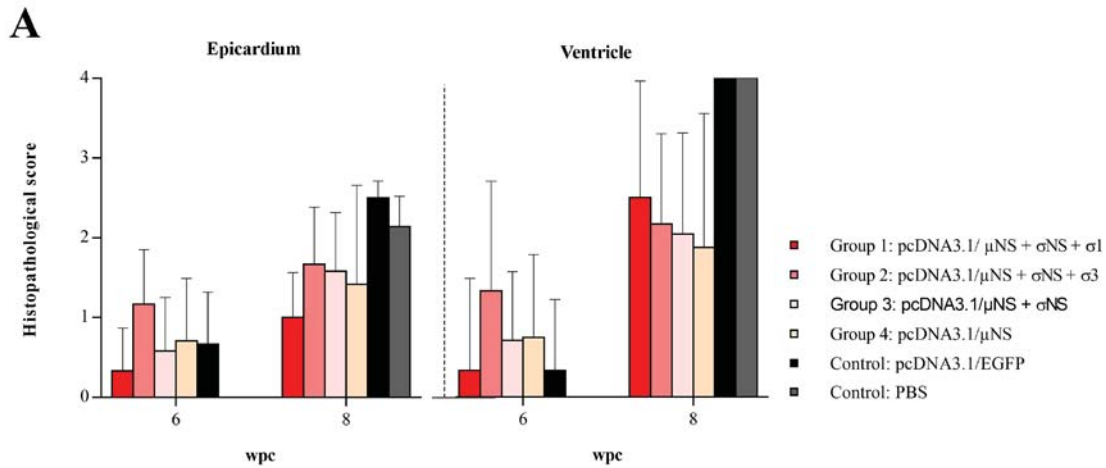


Figure 6. Vaccine trial #II: Piscine orthoreovirus (PRV) RNA load in blood cells and plasma

RT-qPCR of PRV gene segment S1 in blood cells (left) and plasma (right) from cohabitant fish in Group 1 (pcDNA3.1/ μ NS + σ NS + σ 1) and the two control groups (pSAV/EGFP and PBS) in vaccination trial #II. Ct-values of individual fish (dots) and mean (line). The Ct-values from all individuals within the separate groups are compared to the individual Ct-values from the control groups at each timepoint using the Mann Whitney compare ranks test; Star = p - value \leq 0.05. n = 12. wpc = weeks post challenge.



B

Group	6 wpc		8 wpc	
	Epicardium	Ventricle	Epicardium	Ventricle
1	0,3	0,3	1,0	2,5
2	1,2	1,3	1,7	2,2
3	0,6	0,7	1,6	2,0
4	0,7	0,8	1,4	1,9
5	0,7	0,3	2,5	4,0
6	0,0	0,0	2,1	4,0

Figure 7. Vaccine trial #II: Histopathological scores in the epicardium and ventricle

(A) Bars illustrating mean histopathological score in epicardium and ventricle from each group at each timepoint of sampling (wpc) in vaccination trial #II. (B) Table showing mean histopathological score in epicardium and ventricle from each group at each timepoint of sampling. The histopathological scores from all individuals within the separate groups are compared to the individual histopathological scores from the control groups at each timepoint using the Mann Whitney compare ranks test; Star = p - value ≤ 0.05 . n = 12. wpc = weeks post challenge.

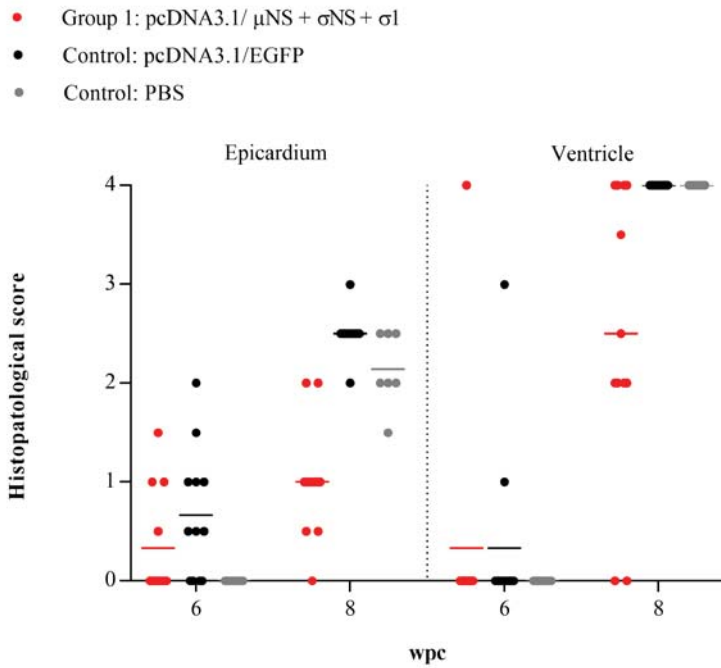


Figure 8. Vaccine trial #II: Histopathological scores in epicardium and ventricle

The individual histopathological scores (dots) and mean (line) in epicardium and ventricle from fish in Group 6 (pcDNA3.1/ μ NS + σ NS + σ 1) and the control groups (pSAV/EGFP and PBS). n = 12. wpc = week post challenge.

Supplementary tables

Table S1. Expression plasmids

Primers used for cloning the full-length ORFs of the ten different PRV segments into the replicon vector pSAV, and the four PRV segments M1, S4, S1 and S3 into the eucaryotic expression vector pcDNA3.1. Start codons are marked in bold and epitope tags for expression are marked in italic.

Plasmid	Primer	Nucleotid sequence (5'→3')
pSAV μ NS	Forward	TCAACCAACCACCGGTGCCACC ATGGCTGAATCAATTA CTTTTGGAGGA
	Reverse	TACCGGAATGGCGCGCCTCAGCCACGTAGCATTATTCAC
pcDNA3.1 μ NS	Forward	GCCGCTCGAGTCTAGAGCCACC ATGGCTGAATCAATTA CTTTTGG
	Reverse	AAACGGGCCCTCTAGATCAGCCACGTAGCATTATTCAC
pSAV σ NS	Forward	TCAACCAACCACCGGTGCCACC ATGTCGAACTTTGATCTTGGACGTCAG
	Reverse	TACCGGAATGGCGCGCCTAACAAAAACATGGCCATGATGCC
pcDNA3.1 σ NS	Forward	GCCGCTCGAGTCTAGAGCCACC ATGTCGAACTTTGATCTTGG
	Reverse	AAACGGGCCCTCTAGACTAACAAAAACATGGCCATGA
pSAV σ 1	Forward	AACCACCGGTGCCACC ATGCATAGATTACCAAGAAGAC
	Reverse	ACCGGAATGGCGCGCCTAGATGATGATCAGGAAGTCTCC
pcDNA3.1 σ 1	Forward	GTTTAAACTTAAGCTT ATGCATAGATTACCAAGAAGAC
	Reverse	CTGGACTAGTGGATCCCTAGATGATGATCAGGAAGTCTCC
pSAV σ 2	Forward	AACCACCGGTGCCACC ATGGCTAGAGCAATTTCTCGGGT
	Reverse	ACCGGAATGGCGCGCCTTACCGTGTATTTGCCGCCATTAG
pSAV σ 3	Forward	AACCACCGGTGCCACC ATGGCGAACCATAGGACGGCGACA
	Reverse	ACCGGAATGGCGCGCCTCAGCCGATGACCATTTGAGCAA
pcDNA3.1 σ 3	Forward	GTTTAAACTTAAGCTT ATGGCGAACCATAGGACGGCGACA
	Reverse	GATATCTGCAGAATTCTCAGCCGATGACCATTTGAGCAA
pSAV μ 1	Forward	TCAACCAACCACCGGTGCCACC ATGGGTAATATCAGACAAGTAACA
	Reverse	TACCGGAATGGCGCGCCTATTTTGGCCTCGACGTGAGTCT
pSAV μ 2	Forward	TCAACCAACCACCGGTGCCACC ATGCCTATCATAAACTGCCAATTG
	Reverse	TACCGGAATGGCGCGCCTCACTACCAGCTGTAGACCACCA
pSAV λ 1	Forward	AACCACCGGGGCCACC ATGAGCGACTTAAGAGGAAAGAT
	Reverse	ACCGGAATGGCGCGCCTTAGTTGAGTACAGGATGAGTCAA
pSAV λ 2	Forward	AACCACCGGTGCCACC ATGGCTACGCTTTATGGCTACGC
	Reverse	ACCGGAATGGCGCGCCTATGGCAACTTTAAGAACGTGTA
pSAV λ 3	Forward	TCAACCAACCACCGGTGCCACC ATGAGAAACCTAAAGCGCTTGTCA
	Reverse	TACCGGAATGGCGCGCCTAAAAATCGGACCATCCAATT
pSAV σ NS N-MYC	Forward	TCAACCAACCACCGGTGCCACC ATGGAACAAAACTCATCT
	Reverse	TACCGGAATGGCGCGCCTAACAAAAACATGGCCATGATGCC
pcDNA3.1 σ NS N-MYC	Forward	GCCGCTCGAGTCTAGAGCCACC ATGGAACAAAACTCATCTCAGAAGAGGATCTG ATGTCGAACTTTGATCTTGG
	Reverse	AAACGGGCCCTCTAGACTAACAAAAACATGGCCATGA
pSAV σ 2 N-HA	Forward	CCACCGGTGCCACCGCCACC ATGTACCATACGATGTTCCAGATTACGCT GCTAGAGCAATTTTCTCGGGTA
	Reverse	ACCGGAATGGCGCGCCTTACCGTGTATTTGCCGCCATTAG
pSAV μ 2 N-HA	Forward	CCACCGGTGCCACCGCCACC ATGTACCATACGATGTTCCAGATTACGCT CCTATCATAAACTGCCAATTG
	Reverse	TACCGGAATGGCGCGCCTCACTACCAGCTGTAGACCACCA
pSAV λ 2 N-HA	Forward	CCACCGGTGCCACCGCCACC ATGTACCATACGATGTTCCAGATTACGCT GCTACGCTTTATGGGTACGCA
	Reverse	ACCGGAATGGCGCGCCTATGGCAACTTTAAGAACGTGTA
pSAV λ 3 N-HA	Forward	CCACCGGTGCCACCGCCACC ATGTACCATACGATGTTCCAGATTACGCT AGAAACCTAAAGCGCTTGTCAA
	Reverse	TACCGGAATGGCGCGCCTAAAAATCGGACCATCCAATT

Table S2. Histopathological scoring criteria

Criteria used for histopathological scoring discriminating between epicardial and ventricular lesions in the heart.

Score	Description: Epicard
0	Normal appearance
1	Focal/multifocal (2 - 4 foci) of inflammatory cells lifting the epicardial layer from the surface of the heart, typically 2 - 3 layers thick.
2	Diffuse infiltration of inflammatory cells (mononuclear) > 5 cell layers thick in most of the epicard present. The infiltration of cells is multifocal to diffuse and can involve parts or the entire epicardium available for assessment.
3	Diffuse infiltration of inflammatory cells (mononuclear) > 10 cell layers thick in most of the epicard present. Moderate pathological changes consisting of moderate number of inflammatory cells in the epicardium.
4	Diffuse infiltration of inflammatory cells (mononuclear) > 15 cell layers thick in most of the epicard present. Severe pathological changes characterized by intense infiltration of inflammatory cells in the epicardium.

Score	Description: Ventricle
0	Normal appearance
1	Vascular changes in the small vessels of the compact layer characterized by enlarged endothelial cells, typically stretching out. Minor inflammatory changes in the compact layer without significant involvement of the spongy layer.
2	Focal to multifocal inflammatory foci (2 - 5 foci) of the compact layer and/or the spongy part (2 - 5 foci). Extension typically seen along small vessels and perivascular infiltration.
3	The changes in the compact layer are multifocal or diffuse in areas and typically concentrated along small blood vessels. Combines with multifocal to diffuse changes in the spongy layer.
4	Widespread to diffuse infiltration of inflammatory cells in the compact layer in a multifocal pattern. Degeneration and/or necrosis of muscle fibers may be/are seen. Atrium can also be involved with inflammatory changes.

Table S3. Plasmid constructs and expression analysis

Five of the plasmids were made with an epitope tag for expression analysis due to lack of available antibodies.

Backbone	Insert	(Tag)	Antibody	Expression
pSAV/pcDNA3.1	μ NS	-	Anti- μ NS	Yes
pSAV/pcDNA3.1	σ NS	(MYC)	Anti-MYC	Yes
pSAV/pcDNA3.1	σ 1	-	Anti- σ 1	Yes
pSAV	σ 2	(HA)	Anti-HA	Yes
pSAV/pcDNA3.1	σ 3	-	Anti- σ 3	Yes
pSAV	μ 1	-	Anti- μ 1C	Yes
pSAV	μ 2	(HA)	Anti-HA	Yes
pSAV	λ 1	-	Anti- λ 1	Yes
pSAV	λ 2	(HA)	Anti-HA	Yes
pSAV	λ 3	(HA)	Anti-HA	Yes

Supplementary figures

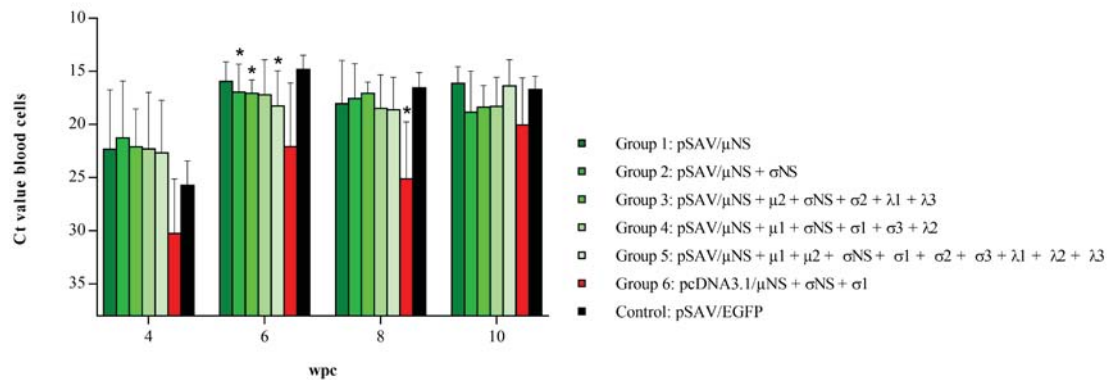


Figure S1. Vaccine trial #I: Piscine orthoreovirus (PRV) RNA load in blood cells

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) of PRV gene segment S1 in blood cells from all fish in vaccination trial #I. Bars illustrating mean Ct-value with SD calculated from each vaccination group per time-point. The Ct-values from all individuals within the separate groups are compared to the individual Ct-values from the control group at each timepoint using the Mann Whitney compare ranks test; Star = p - value ≤ 0.05 . n = 6. wpc = weeks post challenge.

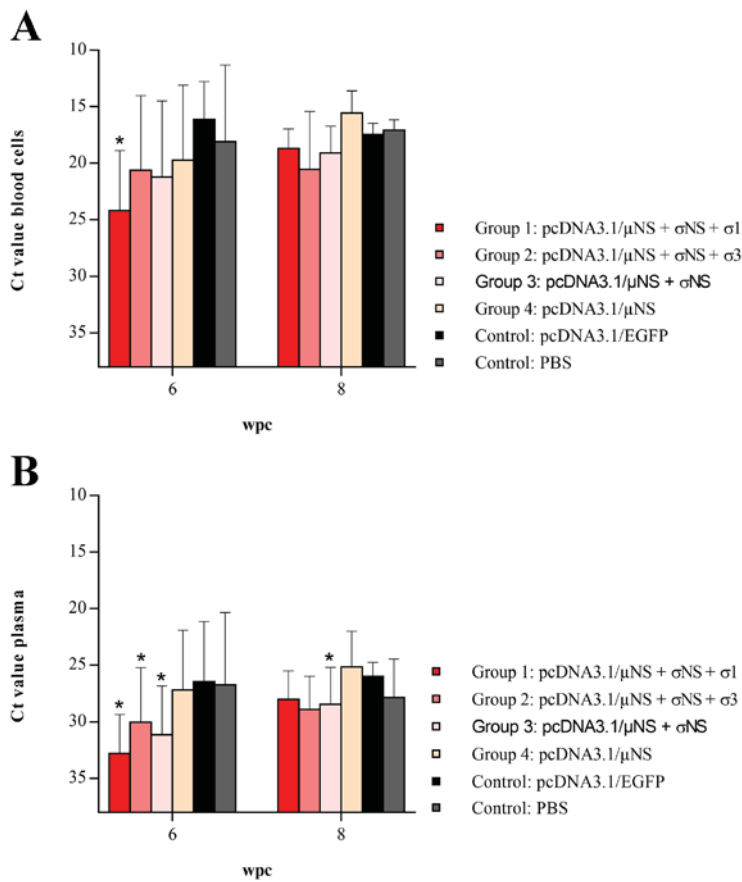


Figure S2. Vaccination trial #II: Piscine orthoreovirus (PRV) RNA load in blood cells and plasma

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) of PRV gene segment S1 in blood cells (A) and plasma (B) from all fish in vaccination trial #II. Bars illustrating mean Ct-value with SD calculated from each vaccination group per time-point. The Ct-values from all individuals within the separate groups are compared to the individual Ct-values from the control group at each timepoint using the Mann Whitney compare ranks test; Star = p - value \leq 0.05. n = 12. wpc = weeks post challenge.

