Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Anatomy Unit

Philosophiae Doctor (PhD)
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Melanised focal muscle changes in Atlantic salmon – Interactions between infection and immunity

Melaniserte fokale muskelforandringer hos Atlantisk laks – Interaksjoner mellom infeksjon og immunitet

Håvard Bjørgen

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3 ABBREVIATIONS

AID – activation induced cytidine deaminase
APC – antigen presenting cell
Arg – arginase
BCR – B cell receptor
BKD – bacterial kidney disease
CD – cluster of differentiation
CMS – cardiomyopathy syndrome
DAMP – danger-associated molecular pattern
DNA – deoxyribonucleic acid
FFPE – formalin-fixed paraffin embedded
HE – haematoxylin and eosin
HSMI – heart and skeletal muscle inflammation
IEL – intra-epithelial leukocyte
Ig – immunoglobulin
IHC – immunohistochemistry
IFN – interferon
I.M. – intramuscular
iNOS – inducible nitric oxide synthase
I.P. – intraperitoneal
IPN – infectious pancreatic necrosis
ISA – infectious salmon anemia
ISH – *in situ* hybridization
NK – natural killer
MALT – mucosa associated lymphoid tissue
MHC I/II – major Histocompatibility Complex class I/II
PAMP – pathogen-associated molecular pattern
PAS – periodic acid-Schiff
PD – pancreas disease
PMCV – *Piscine myocarditis virus*
PRR – pattern recognition receptor
PRV-1 – *Piscine orthoreovirus*
RT-qPCR – reverse transcription quantitative polymerase chain reaction
RLR – rig-like receptor
RNA – ribonucleic acid
SAV – *salmonid alphavirus*
TCR – T cell receptor
TLR – toll-like receptor
TNF – tumour necrosis factor
TyRP – tyrosine-related protein

UV – ultraviolet
WPC – weeks post challenge
16SrRNA – 16S ribosomal ribonucleic acid

4 SUMMARY

Atlantic salmon (*Salmo salar*) farming has increased rapidly in Norway over the last 50 years. However, the large-scale farming of salmon battles serious health problems. There are currently many adverse conditions threatening animal welfare and decreasing profits in the production. Among these threats are infectious diseases, some of which have been controlled with vaccination. Other infectious agents have however proved difficult to control, and therefore, the industry suffers numerous outbreaks of such diseases every year. In addition to infectious diseases, conditions affecting the quality of the end-product, such as melanised focal changes in the fillet, also known as *black spots*, are of major concern to the industry.

The melanised focal changes were recognised as a quality problem in the beginning of the third millennium. From then on, industry representatives have complained that the changes have increased in frequency. As many as 10-50 % of the fillets at the abattoirs have been reported to be melanised, resulting in quality degradation or destruction of the fillet. The economic loss related to the condition is estimated to 0.5-1 billion NOK annually in Norway. The changes commonly occur in the cranio-ventral part of the fillet but can also be seen in other areas such as the dorsal musculature or confined to the red musculature under the skin. The condition was early on linked to vaccination and thought to emerge as the result of erroneous intra-muscular injection or displacement and storage of oil-adjuvant in the muscle. This was believed to attract immune cells including pigment-producing melano-macrophages, causing chronic myositis and focal discoloration in the musculature. The fact that melanised focal changes have never been reported in wild (un-vaccinated) Atlantic salmon supported this hypothesis. However, later studies showed that also un-vaccinated salmon could develop melanised focal changes under farming conditions. Thus, the search for other etiological causes continued which included dietary ingredients, various infectious agents, and several management-related issues. None of these factors alone have been shown to cause melanised focal changes, however, changes in feed composition and certain changes in the management have been shown to affect the prevalence and/or severity of the changes.

Another poorly understood quality concern is the haemorrhagic muscle changes, often referred to as *bleedings.* They are occasionally seen in the fillets at the abattoirs and are observed

as red focal changes, often in the same anatomical region and of similar extent as melanised focal changes. The coinciding placement of red and melanised focal changes has led fish health personnel to the hypothesis that the changes could be related, possibly representing different stages of the same condition. However, no reports on the prevalence, the macro- and microscopic characteristics or the development of red focal changes and their putative transition to melanised focal changes were available prior to this study.

This study commenced by investigating selected groups of slaughter-ready, commercially farmed salmon; wild salmon (broodfish from the river Drammenselven); and experimentally kept salmon (in-house tanks at Matre Research Station, Institute of Marine Research), for the presence of red and melanised focal changes and by analysing these changes for various viruses by IHC and RT-qPCR (Paper I). The results showed that muscle changes were primarily found in the farmed salmon, although one small, melanised area was found in one individual fish from Matre. In the farmed fish, red focal changes were also present, and these were histologically characterised as acute haemorrhagic changes with necrosis and leukocyte infiltrates, but without melano-macrophages. Some of the changes in the farmed fish displayed a transient form between red and melanised pathotypes, linking the two conditions together. The group of wild salmon was completely devoid of pathological changes.

In the search for potential pathogens, only PRV-1 was detected. Analysis of PRV-1 loads in blood revealed that groups with and without muscle changes were PRV-1-positive and the wild fish was the only PRV-1-negative group. The PRV-1 load was also investigated in collected muscle changes and compared with a seemingly unaffected muscle sample from the same individual. Red focal changes contained significantly higher levels of PRV-1 RNA than apparently non-affected areas in the white muscle. The results from melanised focal changes showed similar trends, although not significant. At the protein level, using immunohistochemistry, PRV-1 antigen was detected in both red, melanised and transient changes. PRV-1 antigen was seen mainly in mononuclear cells, presumably mostly macrophage-like cells, but occasionally also in erythrocytes and melano-macrophages. In melanised focal changes, PRV-1 antigen was detected within well-organized granulomas. The consistent finding of viral antigen in red and melanised

changes and within granulomas allowed the assumption that PRV-1 was associated to the development of melanised focal changes and a key feature of the pathogenesis.

The next step was to investigate further the role of PRV-1 in the development of melanised focal changes (Paper II). The concurrent development of PRV-1 infection and melanised focal changes was studied by following a population of farmed fish in a commercial production setting and performing sequential samplings throughout the production period (seven major samplings from transfer to seawater until slaughter). The population was PRV-1-negative prior to sea transfer. At the samplings, the fish were autopsied and tested for PRV-1 infection. Muscle changes were described by macroscopical and histological investigations. The fish gradually became naturally infected with PRV-1. Red focal changes occurred throughout the entire observation period with a low prevalence (4 %) regardless of PRV-1 status. The prevalence of melanised foci increased during the trial. The red focal changes were rather homogenous, but the melanised changes were highly diverse, and a histologic classification system was introduced to grade them. Changes of low macroscopic and histological grades were more prevalent in PRV-1-negative fish, whereas diffuse granulomatous melanised changes (high histologic classification grade) only occurred after PRV-1 infection. The results showed that PRV-1 infection did not initiate the development of red focal changes, but through persisting in the changes and inducing chronic inflammation with melanisation, PRV-1 seemed important in the development of granulomatous melanised changes of high histologic classification grade.

In addition to the sequential field samplings, an experimental infection trial was performed aiming to induce melanised focal changes by injecting PRV-1 (Paper II). Groups of fish received intraperitoneal (i.p.) injection with pelleted blood cells containing high loads of PRV-1 or intramuscular (i.m.) injection with purified PRV-1. Groups were also injected i.p. with heat-inactivated PRV-1, either as PRV-1-infected blood cells or as purified PRV-1. The fish were kept at VESO Vikan Research Facility and the experiment ran for 18 WPC. Six fish were autopsied at a three-week interval. None of the fish in this study displayed any muscle changes, either red or melanised.

The results in Paper I and II pointed towards red focal changes as the primary manifestation of the condition. The role of PRV-1 as the causative agent was still debatable, as the changes had been shown to occur prior to infection (Paper II). Still, PRV-1 could very well be involved in the progression towards melanised focal changes. With the histopathological features examined (Paper I) and further classified (Paper II) and their development and prevalence addressed (Paper II), the immunopathological features of the changes remained uninvestigated. Therefore, in Paper III, red focal changes collected at slaughter (PRV-1 positive) were investigated by IHC targeting various immune molecules and RT-qPCR and ISH for different immune genes. These results were compared with samples of high-grade melanised focal changes (PRV-1 positive), revealing a differentiation in nature between acute (red) and chronic (black) manifestations. In all red changes, infiltrates with mononuclear cells were detected, consisting mostly of antigen-presenting cells, most likely macrophages, identified as MHC class I/II-positive cells, but also of T cells identified as CD3-positive and CD8-positive cells. ISH studies for IgM showed few to moderate amounts of B cells in red focal changes. Trends in the RT-qPCR showed upregulation of genes related to innate immunity in the red changes, whereas genes related to adaptive immunity were upregulated in the melanised changes. An important result was the significant downregulation of the anti-inflammatory cytokine IL10 in all red changes. In addition, IHC targeting replicating PRV-1 (the non-structural protein µNS) showed viral replication *in situ* in both red and melanised changes, demonstrating an on-going viral infection and not only the presence of virus.

Finally, the polarisation status of the macrophages in red and melanised focal changes from both PRV-1 infected and non-infected fish was investigated (Paper IV). Samples collected at regular intervals throughout the seawater production period (material sampled in Paper II) in a commercial farm were analysed by multiplex fluorescent *in situ* hybridization (FISH) and qPCR methods. Classically (M1) and alternatively (M2) activated macrophages were targeted by inducible nitric oxide synthase (iNOS2) and arginase-2 (arg-2) expression, respectively. The cell mediated immunity was also addressed by investigating the expression of MHC-I and CD8. Detection of abundant iNOS2 expressing M1-polarised macrophages in red focal changes demonstrated a pro-inflammatory microenvironment. A high level of co-localisation of iNOS2 and

PRV-1 expression was shown. Co-localisation of PRV-1 and cells expressing CD8+ and MHC-I suggested a targeted immune response taking place in the changes. Melanised focal changes, on the other side, had few iNOS2 expressing cells, but a relatively high number of arginase-2 expressing anti-inflammatory M2-polarized macrophages containing melanin. The presence of numerous M2-polarised macrophages and melano-macrophages in melanised changes is in line with an ongoing healing phase. In samples from uninfected fish, a similar response (with macrophage polarisation) was not detected. Taken together, the results indicate that PRV-1 induces a pro-inflammatory environment with abundant M1 macrophages, which over the course of development shows a polarisation towards an M2 phenotype in melanised focal changes.

Taken together, the histological and immunological characteristics of red and melanised focal changes were described, and the pathotypes were linked as different manifestations of the same condition. This was supported by the finding of transient changes, showing the progression from acute red changes, to transient stage changes, to chronic melanised changes. The results strongly indicate that PRV-1 has an important role in the pathogenesis. The presence of virus was linked to the severity of the changes, as it was a consistent finding in high-grade melanised changes with granulomas. In addition, detection of the non-structural viral protein µNS showed that PRV-1 replicates within the changes, thus persisting and acting as the constant trigger of inflammation. However, PRV-1 does not cause the initial bleedings, and low-grade melanised focal changes may develop in PRV-1 negative fish. Thus, PRV-1 alone is not necessarily the initial cause of the condition. When present, PRV-1 was mainly detected in macrophage-like cells, but also occasionally in melano-macrophages. Infected white muscle fibres were never detected. In-depth investigations on the macrophages showed a polarisation of macrophages from M1 (in acute red changes) to M2 (in chronic melanised changes) type in the presence of PRV-1 infection. This coincided with a targeted cell-mediated immune response within the changes. Even though the investigations in Paper I-IV were essential for the characterization of red and melanised focal changes, an underlying cause of the initial red focal changes could not be established.

5 SUMMARY IN NORWEGIAN (SAMMENDRAG)

Oppdrett av Atlantisk laks (*Salmo salar*) har vært i sterk vekst de siste 50 årene i Norge. Allikevel sloss næringen med alvorlige helseproblemer hos fisken. Flere tapsbringende sykdommer truer dyrevelferden og svekker fortjenesten i produksjonen. Blant de fremste truslene er de infeksiøse sykdommene. Noen av disse er godt kontrollert grunnet omfattende vaksinering, mens andre er relativt ukontrollerte, med gjentagende utbrudd hvert år. I tillegg til de infeksiøse sykdommene, er det flere kvalitetsproblemer som forringer kvaliteten på sluttproduktet, f.eks. misfargete, svarte områder i fileten, også kjent som *melaninflekker* eller *svarte flekker*.

Melaninflekker ble anerkjent som et kvalitetsproblem på tidlig 2000-tall. Problemet har siden den gang økt i omfang. Så mye som 10-30 % av filetene på slakteriet rapporteres å ha melaninforandringer, hvilket resulterer i kvalitetsnedgradering eller i verste fall destruksjon. Det økonomiske tapet for norsk oppdrettsnæring relatert til melaninforandringer er estimert til mellom 0,5-1 milliard årlig. Flekkene finnes vanligvis kranio-ventralt i fileten, men kan også forekomme andre steder, som f.eks. diffust langs ryggmuskulaturen eller avgrenset til den røde muskulaturen rett under huden. Melaninflekker ble først antatt forårsaket av vaksinering, enten som et resultat av feilstikk i muskulaturen eller re-distribusjon og lagring av olje-adjuvans (vaksinehjelpestoff) i fileten. Dette ville medført en kronisk muskelbetennelse, hvor blant annet melano-makrofager blir rekruttert; en type pigment-produserende hvit blodcelle. Pigmentet i betennelsesreaksjonen er årsaken til den svarte misfargningen av fileten. Uvaksinert villaks har heller aldri blitt rapportert å ha melaninforandringer i muskulaturen, hvilket støttet hypotesen om vaksinering som årsak. I strid med hypotesen ble det senere vist at også uvaksinert laks kunne utvikle melaninflekker under oppdrettsforhold. Dermed begynte søket etter andre underliggende årsaker. Fôret som benyttes til oppdrettslaks har lenge vært mistenkt som medvirkende, men også diverse infeksiøse agens og faktorer knyttet til driftsforhold har vært undersøkt. Ingen av disse årsakene har alene vist seg å kunne forårsake melaninflekker, men endringer i fôrkomposisjonen og enkelte faktorer i driftsforhold har vist seg å påvirke frekvensen og/eller alvorlighetsgraden av flekkene.

Røde muskelforandringer i fileten, ofte bare kalt blødninger, har blitt observert med en tilsynelatende lav frekvens på slakterier. Blødningene ser ut som røde, fokale forandringer i

fileten, ofte i samme anatomiske område og med lik utbredelse som melaninflekker. Disse likhetene førte til antagelsen om at blødninger og melaninflekker var relaterte til hverandre, og trolig var ulike manifestasjoner av samme tilstand. Selv om dette var antatt, fantes det ingen publikasjoner på prevalens, makro- og mikroskopiske karakteristikker eller utviklingen av røde, fokale forandringer, ei heller deres mulige forbindelse til melaninflekker.

Dette studiet begynte med å undersøke utvalgte grupper av slaktemoden oppdrettslaks, villaks (fra Drammenselven) og laks holdt i forsøkstanker (ved Havforskningsinstituttet på Matre utenfor Bergen) for tilstedeværelse av røde og svarte flekker og analysere flekkene for forskjellige virus med IHC og RT-qPCR (Artikkel I). Muskelforandringer ble primært funnet i oppdrettslaksen, med unntak av et lite melanisert foci i én fisk fra Matre. Røde flekker ble karakterisert med histologiske undersøkelser og beskrevet som akutte muskelforandringer med blødninger, vevsdød og infiltrater av betennelsesceller, dog uten melano-makrofager. Enkelte muskelforandringer i oppdrettslaksen lignet såkalte overgangsforandringer med innslag av både blod og melanin, hvilket tyder på en progresjon i utviklingen fra røde til svarte flekker. Gruppen med villfisk var uten patologiske forandringer.

I søket etter infeksiøse agens ble det kun funnet PRV-1. Analyser av PRV-1-RNA i blodet viste at grupper både med og uten muskelforandringer var PRV-1-positive. Gruppen med villfisk var den eneste gruppen som var PRV-1-negativ. PRV-1-RNA ble også målt i muskelforandringene og sammenliknet med uaffiserte muskelprøver tatt fra samme fisk. Røde forandringer inneholdt signifikant høyere nivåer PRV-1-RNA enn uaffiserte muskelprøver. Resultatene fra svarte flekker viste samme trend, dog ikke signifikante forskjeller. På proteinnivå, ved å bruke immunhistokjemi, ble PRV-1-antigen påvist i både røde, svarte og overgangsforandringer. PRV-1-antigen var til stede hovedsakelig i mononukleære celler, antageligvis mest makrofag-liknende celler, men av og til også i røde blodceller og melano-makrofager. I svarte flekker ble PRV-1-antigen påvist i granulomer. Tilstedeværelse av PRV-1-antigen i alle røde og svarte flekker, og også i granulomer, tillot antagelsen om at PRV-1 var assosiert med utviklingen av svarte flekker og trolig en viktig faktor i patogenesen.

Det neste steget var å ytterligere undersøke PRV-1s rolle i utviklingen av svarte flekker (artikkel II). Den parallelle utviklingen av PRV-1-infeksjon og svarte flekker ble studert ved å følge en populasjon oppdrettslaks gjennom en normal, kommersiell produksjonsfase i sjø, med hyppige uttak og registreringer i perioden (feltforsøk med syv store uttak fra overføring til sjø frem til slakting). Fiskepopulasjonen var PRV-1-negativ før utsett. Ved hvert uttak ble fisken obdusert og test for PRV-1-infeksjon. Muskelforandringer ble beskrevet makroskopisk og histologisk, og et klassifiseringssystem ble etablert. Populasjonen ble gradvis PRV-1-infisert. Røde flekker forekom gjennom hele forsøksperioden med en stabilt lav prosent (ca. 4%), uavhengig av PRV-1-status. Prevalensen av svarte flekker økte i den samme perioden. Forandringene (svarte flekker) viste stor diversitet: forandringer av lav makroskopisk grad og histologisk kategori forekom oftere i PRV-1-negativ fisk, mens diffust granulomatøse og melaniserte forandringer kun forekom i PRV-1-infisert fisk. Disse resultatene viste at PRV-1-infeksjon ikke initierer utviklingen av røde flekker, men har trolig en viktig betydning for utviklingen av granulomatøse, melaniserte forandringer, ved å persistere i forandringene, med indusering av kronisk betennelse og melanisering som resultat.

I tillegg til feltforsøket, ble det gjennomført et eksperimentelt smitteforsøk med mål om å indusere svarte flekker ved å injisere PRV-1 (artikkel II). Grupper med fisk ble injisert i.p. med pelletert blod med høye nivåer PRV-1 eller i.m. med renset PRV-1. Grupper ble også injisert i.p. med varmeinaktivert PRV-1, enten som PRV-1-infiserte blodceller eller renset PRV-1. Forsøket gikk på VESO Vikan forskningsstasjon over 18 uker. Seks fisk ble obdusert med tre ukers intervall. Samlede resultater fra forsøket viste at ingen av gruppene utviklet muskelforandringer, hverken røde eller svarte.

Resultatene fra artikkel I og II pekte mot røde flekker som den innledende manifestasjonen av tilstanden. Med de histopatologiske forandringene beskrevet (artikkel I) og med frekvensen kartlagt (artikkel II), var fortsatt de immunopatologiske egenskapene ukjente. Derfor, i artikkel III, ble røde flekker fra slaktemoden fisk undersøkt med RT-qPCR, ISH og IHC for forskjellige immungener og molekyler. Resultatene ble vurdert opp mot svarte flekker for å sammenligne akutte og kroniske stadier av tilstanden. I alle røde forandringer ble det funnet infiltrater av mononukleære celler, hovedsakelig MHC klasse I/II-positive celler, men også T-celler (CD3- og

CD8-positive). ISH mot IgM viste få til moderate mengder B-celler i røde forandringer. Trender i RT-qPCR-resultatene viste oppregulerte gener relatert til medfødt immunitet i røde flekker, mens gener relatert til adaptiv immunitet var oppregulerte i svarte flekker. Et viktig resultat var den signifikante nedreguleringen av det anti-inflammatoriske cytokinet IL10 i alle røde flekker. I tillegg ble det vist en pågående replikering av virus i både røde og svarte flekker, ved at det ikke-strukturelle proteinet µNS ble visualisert med IHC. Proteinet uttrykkes kun under virusreplikasjon, og resultatet viser derfor at viruset ikke bare er til stede i forandringene, men at det også replikerer, hvilket kan forklare den kroniske manifestasjonen.

I den videre karakteriseringen av immunresponsen, ble makrofagpolariseringen undersøkt i røde og svarte flekker fra henholdsvis PRV-1-infisert og ikke-infisert fisk (artikkel IV). Prøver innsamlet ved jevne mellomrom under hele sjøfasen (deler av samme materiale i artikkel II) ble undersøkt med multipleks *in situ* hybridisering og qPCR. Klassisk (M1) og alternativt (M2) aktiverte makrofager ble visualisert med prober mot henholdsvis inducible nitric oxide synthase (iNOS2) og arginase-2 (arg-2). Den cellemedierte immunresponsen ble også kartlagt ved å undersøke uttrykket av MHC-I og CD8. Et stort antall proinflammatoriske, iNOS2-positive M1-makrofager ble funnet i røde flekker (PRV-1-postiv fisk). En nesten perfekt samlokalisering mellom iNOS2 og PRV-1 ble vist. Samlokalisering mellom PRV-1 og CD8- og MHC-I-uttrykkende celler var også tydelig, hvilket tyder på en målrettet immunrespons, antageligvis mot virus, i flekkene. Svarte flekker hadde derimot få iNOS2-positive celler, men et relativt høyt antall anti-inflammatoriske arginase-2-positive makrofager (M2), både med melanin og uten. Tilstedeværelsen av disse M2-makrofagene i svarte flekker stemmer overens med en pågående helingsprosess. Polarisering av makrofagene ble ikke påvist i forandringer fra PRV-1-negativ fisk. Oppsummert indikerer resultatene I artikkel IV at PRV-1 induserer et pro-inflammatorisk miljø med et stort antall M1-makrofager, som over tid viser en polarisering mot M2-type og avheling i svarte flekker.

I dette studiet har histologiske og immunologiske karakteristika i røde og melaniserte fokale forandringer blitt beskrevet. Det ble vist at de to patotypene (rød og melanisert flekk) representerer ulike manifestasjoner av samme tilstand. Dette ble understøttet av funnet av overgangsformer, som viste en progresjon i utviklingen av tilstanden, som starter som akutte,

røde forandringer, og videre blir til overgangsforandringer som til slutt ender i kroniske, melaniserte forandringer. Resultatene indikerer sterkt at PRV-1 har en viktig rolle i patogenesen. Tilstedeværelsen av virus ble satt i sammenheng med forandringenes alvorlighetsgrad, da PRV-1 var et gjennomgående funn i alvorlige (høygradige) melaniserte forandringer med granulomer. I tillegg ble det ikke-strukturelle virusproteinet µNS detektert, hvilket betyr pågående virusreplikasjon i forandringene, som igjen kan forklare den persistente infeksjonen. Viruset blir ikke brutt ned og fjernet av immunforsvaret, men er trolig pådriveren og årsaken for den lokale betennelsesreaksjonen. Det ble dog vist at røde fokale forandringer og lav-gradige melaninflekker kan oppstå uten PRV-1-infeksjon, hvilket betyr at PRV-1 ikke er den primære årsaken til blødninger. Når PRV-1 var til stede, ble viruset funnet i makrofag-liknende celler, men av og til også i melano-makrofager. Infiserte hvite muskelfibre ble aldri påvist. I en videre undersøkelse av immunresponsen ble det funnet en polarisering av makrofagene fra M1- (i akutte, røde forandringer) til M2-type (i kroniske, melaniserte forandringer) i PRV-1 infisert fisk. I tillegg ble det påvist en målrettet celle-mediert respons i forandringene, trolig mot PRV-1. Selv om undersøkelsene i artikkel III og IV var essensielle for kartleggingen av de immunopatologiske egenskapene i røde og svarte flekker, er den underliggende årsaken til røde flekker fortsatt ukjent. Allikevel tyder resultatene i Artikkel I-IV på at PRV-1 har en viktig rolle for utviklingen og alvorlighetsgraden av svarte flekker.

6 LIST OF PAPERS

Paper I

Bjørgen H, Wessel O, Fjelldal PG, Hansen T, Sveier H, Sæbø HR, Enger KB, Monsen E, Kvellestad A, Rimstad E, Koppang EO. *Piscine orthoreovirus (PRV-1) in red and melanised foci in white muscle of Atlantic salmon (Salmo salar).* Published in Veterinary Research, 2015, 46:89

Paper II

Bjørgen H, Haldorsen R, Oaland Ø, Kvellestad A, Kannimuthu D, Rimstad E, Koppang EO. *Focal melanised changes in skeletal muscle in farmed Atlantic salmon after natural infection with Piscine orthoreovirus (PRV-1).* Published in Journal of Fish Diseases, 2019, 42(6):935-945

Paper III

Bjørgen H, Kumar S, Gunnes G, Press C, Rimstad E, Koppang EO.
Characterization of important immune parameters in red focal changes in Atlantic salmon (Salmo salar) white muscle. Published in Veterinary Immunology and Immunopathology, 2020, 222:110035

Paper IV

Malik SM, **Bjørgen H**, Nyman IB, Wessel Ø, Koppang EO, Dahle MK, Rimstad E.
M1 polarized macrophages in red and black spots in white muscle of Atlantic salmon (Salmo salar) are PRV-1 infected and cause a pro-inflammatory environment. Published in Frontiers in Immunology, 2021, 12:664624

7 INTRODUCTION

7.1 BACKGROUND FOR THIS THESIS

7.1.1 Fish farming in Norway

Fish farming is an important industry in Norway. Farmed Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) are the two leading aquaculture species where Atlantic salmon makes up 95 % and rainbow trout about 4.5 % of the total volume [1]. Norway is the second largest exporter of seafood (in terms of value) behind China. The total value of the 2.7 million tons Norwegian seafood, including wild caught and farmed species exported in 2019, was 107.3 billion NOK. Farmed Atlantic salmon was by far the most important species as measured in both volume and value accounting for 1.1 million tons and 72.5 billion NOK, respectively [2].

The geography of the Norwegian coastline ensures suitable conditions for salmon farming with cool water temperatures and fjords shielded from heavy waters. Atlantic salmon can endure temperatures between 0-25 °C but the optimum is around 16 °C in the sea-living phase [3]. The production line of farmed salmon is a fast-forward version of the life cycle of the wild salmon starting in freshwater and terminating in the sea (**Figure 1**). Broodfish farms ensure the supply of fertilised eggs. After hatching, the alevins (yolk sac larvae) feed on their yolk sac until the beginning of exogenous feeding as fry. They subsequently go through the parr stage to reach smoltification; a pre-adaptation to the environmental changes in seawater. When ready, the fish are transferred to seawater cages. Under farming conditions, the fingerling and the seawater periods are considerably shortened to the comparative periods in the wild. The production period from fertilised egg to slaughter takes between 24-36 months. The intensified production, including the number and density of fish in the farms, has brought numerous health problems, and especially ectoparasites and viral diseases are of major importance. Most of the currently used vaccines efficiently target bacterial diseases whereas immunisation against viral diseases has so far been less successful.

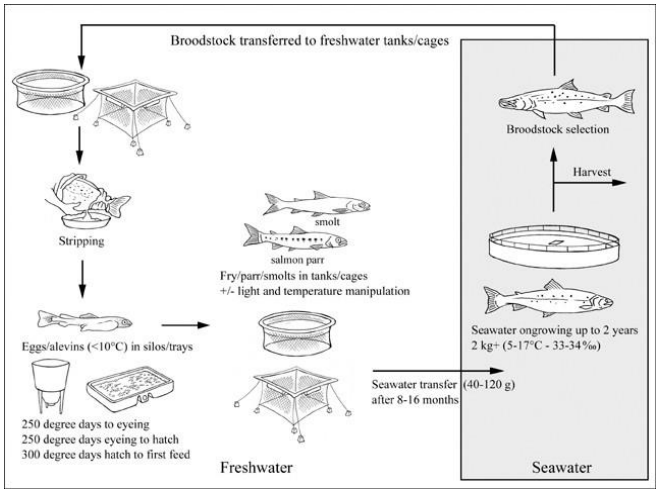

Figure 1. Production cycle of farmed Atlantic salmon. Broodstock fish are selected and transferred to freshwater tanks. Eggs are stripped and fertilized with milt followed by eyeing of the eggs and hatching. Alevins are transferred to freshwater tanks. After yolk sac absorption, the alevins receive their first feed. They subsequently go through the parr stage to reach smoltification. Both small (40-120 g) and bigger (120 g+) smolts can be transferred to sea. Extensive growth is obtained during the seawater period. Slaughter is normally after one-two years at sea when the fish has reached a weight of 4-6 kg. Vaccination is carried out in the parr stage in freshwater before smoltification. Source: www.fao.org

7.1.2 Melanised focal changes

The most serious problem affecting the quality of the fillet of Atlantic salmon is the discoloured changes known as *black spots* or *melanised spots* (**Figure 2A**). These changes are found on average in 20 % of the fillets produced [4] and are thought to occur mostly in the cranio-abdominal region of the fillet. The changes are sporadically seen as more red than black and have in such cases been referred to as *haemorrhages* or *bleedings* (**Figure 2B**). Information on such haemorrhagic changes is very limited due to their low prevalence, but it has been hypothesized that the haemorrhages are linked to the development of black spots. The affected areas of the fillets are trimmed, resulting in a fillet of lower quality and value. The estimated economic loss is between 0.5-1 billion NOK annually [4].

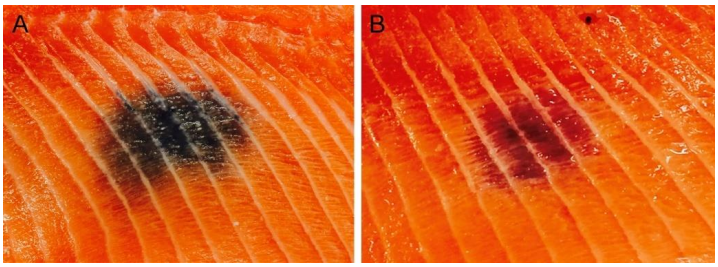

Figure 2. Melanised (A) and red (B) focal change in the fillet of farmed Atlantic salmon. Photo: DVM Håkon Rydland Sæbø.

The aetiology of the condition appears complex; however, the reason for the discoloration is attributed to the presence of melano-macrophages, a specialized immune cell in ectothermic vertebrates appearing to be capable of pigment production [5]. When pigment accumulates locally in the muscle, it turns black, *i.e.,* a black spot. Throughout this thesis, black spots and haemorrhages are referred to as *melanised focal changes* and *red focal changes*, respectively.

For the characterisation of the pathological characteristics of these changes, the anatomical considerations and the immune responses must be considered and evaluated. Importantly, the responses of the pigmentary system and its links to the immune system need to be addressed. As a result, this thesis is a synthesis of several broad scientific disciplines undertaken to increase the knowledge on melanised focal changes. To provide a background on this multi-disciplinary challenge, the introduction covers the following topics:

- The general health considerations of farmed salmon in Norway
- Viral diseases affecting the musculature of salmon with emphasis on HSMI
- A brief overview of the anatomy of the salmonid muscle.
- The teleost immune system with emphasis on different immune cells and their response to viral infection
- The teleost extracutaneous pigmentary system with emphasis on melano-macrophages and their role in melanised focal changes

7.2 GENERAL HEALTH CONSIDERATIONS OF FARMED SALMON IN NORWAY

The accelerating growth of the salmon farming industry in Norway has increased the attention on fish health. Salmon kept under farming conditions are protected by the Norwegian Animal Welfare Act [6], promoting good animal welfare and general respect for animals. Further requirements for aquaculture facilities are described in the national regulations.

There are numerous health problems affecting farmed salmon in Norway. Among these, the infectious diseases are especially challenging. Infectious diseases are common in all intensive animal husbandries and farmed salmon is no exception. Infectious diseases are caused by organisms — such as parasites, fungi, bacteria, or viruses, which grow and multiply in the fish. Most infectious diseases are complex of nature with an outcome dependent on multiple factors. These include the agent itself, the host and the environmental conditions (known as the causal triad model [7]). Parasites have been problematic in Norwegian salmon farms for a long time, especially the recurrent infections with salmon sea lice (*Lepeophtheirus salmonis*). Historically, bacterial diseases have been one of the major hurdles for salmon farming, but the most threatening diseases were overcome by vaccination. On the viral side, there are several diseases occurring with a high frequency each year (**Table 1**) [8]. Infectious salmon anaemia (ISA), Cardiomyopathy syndrome (CMS), Pancreas disease (PD), Infectious pancreatic necrosis (IPN) and Heart and skeletal muscle inflammation (HSMI) are according to health surveys the five most significant viral diseases in Norwegian farmed salmon. Thus, viral diseases are currently regarded as a main health concern, in addition to sea lice infections. As *Piscine orthoreovirus* (PRV-1), the etiological agent causing HSMI, is the main virus of interest in this study, an in-depth background on PRV-1 and HSMI is given below.

	2016	**2017**	**2018**	**2019**	**2020**
ILA	12	14	13	10	23
PD	138	176	163	152	158
HSMB	101	93	104	79	161*
IPN	27	23	19	23	22*
CMS	90	100	101	82	154*

Table 1. Viral disease outbreaks in Norwegian salmon farms from 2016 to 2020. *For 2016-2020, the number of positive farm sites is based on samples sent to the Norwegian Veterinary Institute. For 2020, data from private laboratories are included.

7.3 PISCINE ORTHOREOVIRUS (PRV-1) AND HEART AND SKELETAL INFLAMMATION (HSMI)

7.3.1 *PRV-1*

PRV-1 belongs to the Reoviridae family, genus *Orthoreovirus*. It is a non-enveloped virus with a double stranded (ds) RNA genome of ten segments encoding at least eight structural and two non-structural proteins (**Figure 3**) [9, 10]. PRV-1 is ubiquitous in farmed salmon in Norway in the marine phase [11] and is also detected in wild salmon in areas with intensive fish farming [12, 13]. PRV-1 has been reported in farmed Atlantic salmon in Canada, Chile, Ireland and Germany [14-18]. PRV-1 has also been described in farmed rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and Coho salmon (*Oncorhynchus kisutch*) [13, 19, 20].

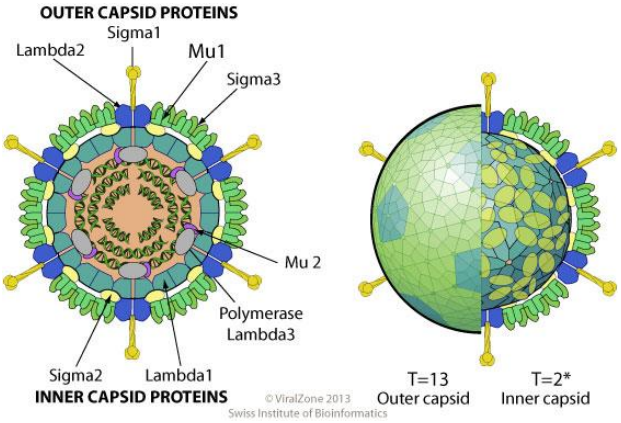

Figure 3. The structure of *orthoreovirus*. Non enveloped, icosahedral virion with a double capsid structure. The different viral proteins are marked. (Source: ViralZone:www.expasy.org/viralzone, SIB Swiss Institute of Bioinformatics).

PRV-1 infects various organs and cells in salmon; however, erythrocytes are important target cells [21]. Since piscine erythrocytes are nucleated, they contain the functional organelles and the transcriptional machinery needed for viral replication and assembly of new viral particles.

For PRV-1, viral replication and assembly have been shown to occur in viral factories seen as globular inclusions in the cytoplasm of erythrocytes [22]. In infected fish, the virus can be found in every blood perfused organ. PRV-1 has also been described in intravascular leukocyte-like cells, in hepatocytes and in cardiomyocytes of the heart ventricle [23, 24]. The latter is of special importance as heart changes are typical for HSMI. It is likely that replication of PRV-1 in cardiomyocytes induces epicardial and myocardial necrosis and inflammation [23]. This is also thought to occur in skeletal muscle cells, primarily affecting the red musculature.

7.3.2HSMI

HSMI was first described in Atlantic salmon in 1999 and is currently one of the most frequent and challenging viral diseases in farmed salmon in Norway [25]. PRV-1 was first identified in HSMI diseased fish by next-generation sequencing (NGS) in 2010 [9] and was later shown by Wessel and co-workers to be the causative agent of the disease [26]. Although causative, a recent study on different PRV-1 isolates showed that they vary in their ability to induce HSMI [27]. This could also explain the sometimes-unexpected outcome of PRV-1 infection without clinical signs of HSMI. Even though HSMI varies in severity, the disease occurs in farmed Atlantic salmon along the entire Norwegian coastline and outbreaks are typically seen 5-9 months after seawater transfer [25]. Clinical signs include appetite loss, lethargy and a mortality rate ranging between 0 – 20 %. Autopsy can reveal typical signs of circulatory failure with congestion of blood in the liver and spleen as well as ascites. Haemorrhages can occur in the liver, the heart and in adipose tissue [25]. The fish can suffer severe heart changes without showing clinical symptoms and without an elevated mortality rate. Thus, the prevalence of HSMI-induced pathological changes is probably higher than reported numbers of disease outbreak. An additional factor that blurs the number of clinical cases is that HSMI was removed from the list of notifiable diseases in 2014, resulting in incomplete numbers. National numbers from 2020 seem more certain, as numbers from private laboratories have been added to the statistics (see **Table 1**). A recent investigation also highlighted the difference in virulence between strains of PRV-1 [27], which is so far not considered in the routine diagnostics.

In farmed rainbow trout, a disease with pathological changes in line with a milder variant of HSMI has been described [28], where the etiological virus is a subtype of PRV termed PRV-3 [19, 29].

7.4 MUSCULATURE IN SALMONIDS – ANATOMICAL, PATHOLOGICAL AND IMMUNOLOGICAL CHARACTERISTICS

A thorough understanding of normal tissue structure and microanatomy is required to recognize and interpret pathological and immunological changes in the skeletal muscle of salmonids. The muscular system consists of cells with contractile abilities, hence capable of generating movement [30]. These movements can be voluntary or involuntary. Overall, there are three different types of musculature: smooth-, heart- and skeletal musculature [30]. Smooth musculature is, as in mammals, involuntary and non-striated and is predominantly found in the walls of hollow organs and in the walls of passageways such as the arteries and veins [31]. Heart musculature constitutes the main tissue of the heart. It is also involuntary, but striated. In comparison, the salmonid heart has a simpler anatomy than the mammalian heart, constructed like a tube with a single atrium leading into a single ventricle [31]. The ventricle is further divided into two separate layers: the inner *stratum spongiosum* lined by the endothelium and the outer *stratum compactum* lined by the epicardium. The stratum spongiosum is made up of loose muscular trabeculae while the stratum compactum consists of heart muscle cells in a circumferential arrangement [31]. Some also recognize the bulbus arteriosus as the final compartment of the heart, consisting mainly of elastic tissue, smooth muscle cells and collagen, however, the bulbus arteriosus can also be regarded as the beginning of the ventral aorta [32]. Finally, skeletal musculature of voluntary and striated character is found on the head- and gill region, the fins and the body (the segmental musculature of the fillet) [31]. In salmonids, two kinds of skeletal musculature are recognised: red and white (more under 7.4.1). The lateral musculature, commonly known as the *fillet*, is proportionally the largest collection of skeletal muscle and it is also the final product for human consumption. Additionally, the skeletal muscle of the fillet is the focus of the investigations in this thesis.

7.4.1 Skeletal muscle anatomy

In mammals, the structure of skeletal muscle is usually divided into tendonous or fibrous parts and muscular parts [30]. The fibrous parts separating the muscle cells can be further sub-divided

based on its location: the epimysium is the outer layer covering the entire muscle, the perimysium covers the fascicle containing bundles of muscle fibres (also called muscle cells or myocytes) and the endomysium is the thin connective tissue lining between individual muscle fibres [30]. The muscle fibres can be classified into fibre type according to their physiological characteristics, in particular slow twitch (type 1) versus fast twitch (type 2). The fast twitch fibres are further subclassified based on differential myosin heavy chain (MYH) gene expression (2A, 2X and 2B) [33].

The anatomy of mammalian skeletal musculature is not easily transferred to the skeletal muscle of fish, although some general features are conserved throughout vertebrate evolution. The musculature of the body, the fins, and the head- and gill region is mainly of skeletal nature. The main musculature of the body is segmentally organized in *myotomes* (a collection of multiple muscle cells), which are separated by thin connective tissue compartments named *myosepta* [31]. The upper part of the musculature, towards the dorsal midline, is termed *epaxial*, while the lower part towards the ventral abdominal region is termed *hypaxial* [31]. The myotomes have the shape of a horizontally flipped W with the lower end directed caudally towards the tail fin. The W segments are organized so that they are stacked together, only separated by myosepta [31]. The myosepta can also be named the intermyotomal facia [34].

In salmon, there are two types of muscle fibres: red (*muscularis lateralis superficialis*) and white (*muscularis lateralis profundus)* [31] (**Figure 4**). Red fibres are located laterally, centred around the lateral line, and make up less than 10 % of total muscle mass. They are enduring and relatively slow. The red fibres have short diameter, are rich in mitochondria and are surrounded by a dense capillary network. They also contain myoglobin and stored energy (glycogen and fat). These features show adaptation towards aerobic metabolism [31].

White muscle fibres make up the main portion of the fillet and are located medially to the red musculature [31]. The white muscle fibres generate speed and are rapid of nature, but their lasting capability is low. In contrast to red fibres, the blood supply to white muscle fibres is poor. The ability to mobilize energy fast generates great force and shows an extreme anaerobic capability. The white muscle fibres are about 5-10 times bigger in diameter than red fibres,

consisting of myofibrils with glycogen and scattered mitochondria [8]. In both red and white muscle, the nuclei of each fibre lie peripherally, immediately below the sarcolemma [34].

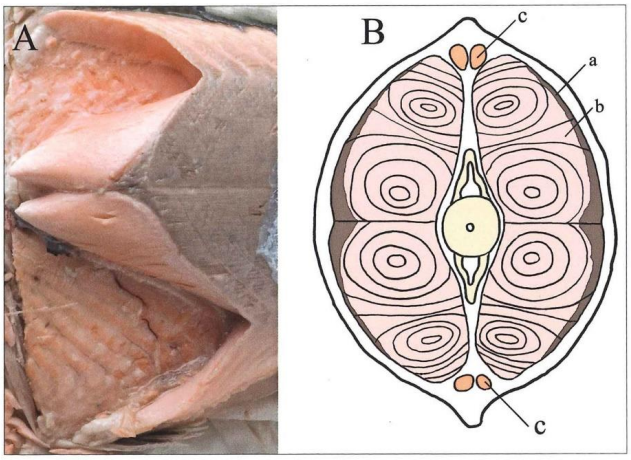

Figure 4. A) Dissection of a heat-treated salmon showing the W-shaped muscle segment (the myotome) of the white muscle. The lateral red muscle is gray/brown. B) Cross section of the tale, schematic figure. The outer red muscle (a) covers the inner white muscle (b). The carinal muscles are indicated (c). Courtesy of prof. em. H. Kryvi.

7.4.2 Pathological responses

General myopathology in salmonids

There are numerous causes of pathological changes in the musculature of salmonids. In wild fish, many metazoan parasites favour the skeletal muscle [35]. However, in farmed salmon, the most common diagnoses are by far PD and HSMI [8], where histopathological changes in the musculature are central in the diagnostic work [25]. Although histological examination is an important tool in this respect, it has several limitations, and additional approaches may be required to reach a diagnosis.

When assessing skeletal muscle damage in salmonids, both red and white muscle should be investigated. One of the first indications of pathological change is the central migration of nuclei [35]. Structural changes of the sarcolemma are usually evident, and eventual haemorrhage can

be seen as extravascular erythrocytes in the endomysium [35]. Oedema, i.e., fluid retention in a tissue, is not uncommon. This is usually followed by degenerative and inflammatory changes. Myophagocytosis, i.e. phagocytosis of muscle cells, is a very common histological trait, which is seen as macrophage-like cells infiltrating necrotic muscle cells [35]. Chronic muscle changes, e.g. caused by persistent infectious agents, are commonly associated with extensive fibrosis, granulomatous inflammation and necrosis, and these histopathological changes may all be present in the tissue simultaneously [35].

The regenerative capacity of skeletal muscle is great. This process is dependent on the degree of damage, but also temperature will affect the host response [35]. Muscle fibre regeneration takes place from stimulated satellite stem cells located beneath the basement membrane of the sarcolemma [35]. The satellite cells are quiescent during homeostasis, but following trauma, the cells activate and divide and generate daughter myoblasts that differentiate and repair the muscle damage [36].

Histopathological changes in HSMI

The characteristic histopathological changes in HSMI are mainly found in the heart and in red skeletal muscle [25]. The first lesions commonly occur in the *stratum compactum* of the ventricle. Typically, perivasculitis of coronary vessels is evident [37]. Focal myocarditis is not uncommon, and a highly cellular epicarditis can also be observed. Subsequently, these cardiac changes spread to affect the entire myocardium leading to a panmyocarditis (**Figure 5A**) [37]. Multifocal necrosis accompanied by severe inflammation, predominantly with neutrophils and macrophages, is seen in the entire ventricle. The atrium is often less affected, however, changes similar to those in the stratum spongiosum might be observed [37]. Necrotic changes in the liver can occur as a result of blood congestion, in addition to tissue oedema [25]. The red skeletal muscle is commonly affected by extensive inflammation and multifocal necrosis (**Figure 5B**) [25]. Degenerative changes and inflammation have also been described in the myocytes of white muscle [25], however, changes in the heart and in the red musculature (in addition to PRV-1 detection by RT-qPCR) are considered typical for the disease.

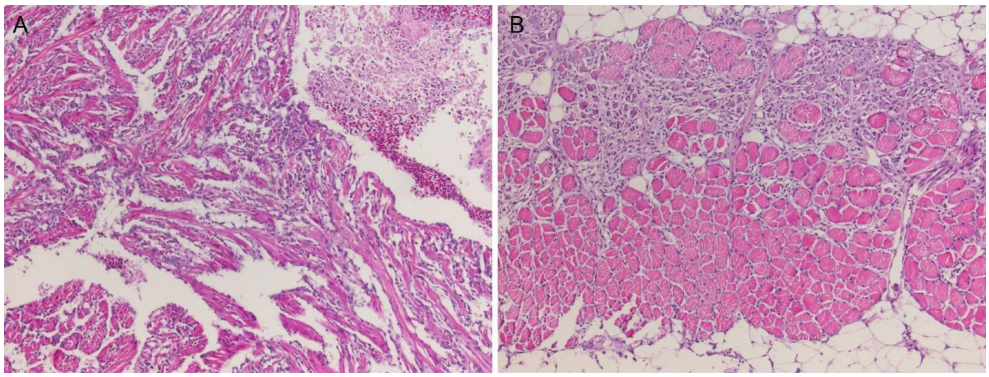

Figure 5. Histopathological changes in HSMI-diseased fish. A) Inflammatory cell infiltrates of the stratum spongiosum of the heart ventricle. B) Degeneration and inflammation in red skeletal muscle. Images: Håvard Bjørgen.

7.4.3 Immunological responses

Overview – innate and adaptive immunity

From early on in evolution, organisms have needed to protect themselves against intruding pathogens and thus the development of inherent defence mechanisms has evolved. These comprise the different components of the immune system with its immune organs, -cells and immuno-active mediators. In vertebrates, the immune system can be divided into an innate (or non-specific) and an adaptive (or specific) part [38]. When addressing ectothermic vertebrate evolution, jawless fish are considered as the primitive ancestor of bony fish, and the development of jaws also coincides with the development of adaptive immunity as we know it in vertebrates [39]. Agnathans have been found to assemble diverse lymphocyte antigen receptor genes through the genomic rearrangement of leucine-rich repeat (LRR)–encoding modules [40, 41] and the cell surface receptors are termed variable lymphocyte receptors (VLRs). However, an immune system based on the adaptive properties of receptors of the immunoglobulin superfamily first emerged in jawed vertebrates [42].

Importantly, the melanin system has been shown to be intertwined with innate immunity, a connection which can be observed phylums such as Arthropoda [43]. In insects, melanin has crucial immunomodulatory functions, and upon interaction with microorganisms and parasites, the production of melanin is an essential component of the innate immune defence [44]. The link

between immunity and pigment production has also been subject for investigation in teleosts [45]. In the spleen of higher teleosts, clusters of pigmented cells accompanied by lymphocytes and macrophages have been suggested to represent the primitive analogues of the germinal centres of lymph nodes of birds and mammals [46-48] (more details about the melanin system are presented in chapter 7.5).

In general, the innate immune system detects pathogens by germline-encoded receptors that act rapidly upon intrusion of exogenous agents [38]. A vast array of pattern recognition receptors (PRRs) recognizes both extra- and intracellular pathogens by responding to danger- or pathogen- associated molecular patterns (DAMPs or PAMPs), e.g. viral RNA [38]. Important PRRs include the toll-like receptors (TLRs) in the cellular and endosomal membranes, and cytoplasmic receptors such as retinoic acid-inducible gene I (RIG-1) like receptors (RLRs) [38]. The innate immune responses seem to be play a crucial role in disease resistance in fish, as their adaptive responses are generally slow [49]. Although slow, the adaptive responses are more specific than the innate response. This is due to antigen-specific receptors, i.e. T cell receptor (TCR) and B cell receptor (BCR) in T and B cells, respectively, generated by selection of specific gene rearrangements following the first encounter with a pathogen [38]. It has long been believed that fish lack antibody affinity maturation, in part because of the absence of germinal centres [42]. However, all fish species investigated to date do have the immunoglobulin mutator activation-induced cytidine deaminase or AID [50], and somatic hypermutation-caused affinity maturation does take place. The increase in affinity appears to be modest as compared with similar reactions in homeothermic vertebrates [50]. Class switch recombination, which is related to increase in affinity, has so far proved absent in fish [50]. Nonetheless, fish can generate adequate adaptive immune responses over time, an immunological principle taken advantage of in vaccination [51].

Many cell types bridge the two branches of the immune response, either with innate or adaptive functions or with functional characteristics from both systems. The immune system represents a very complex interacting network with pro-inflammatory and anti-inflammatory mediators. The communication between the innate and adaptive immune systems involves a multitude of cell-cell interactions in relation to antigen presentation or soluble molecules such as cytokines or chemokines.

Immune organs of fish

In terms of overall construction, the immune system of bony fishes or teleosts is analogous to that of mammals [39, 42, 52]. However, certain structural differences should be highlighted. In contrast to higher vertebrates, fish lack red bone marrow and lymph nodes. The presence of lymphatic vessels is disputed [53]. Primary lymphoid organ functions, i.e., lymphopoiesis, are confined to the kidney, and mostly in the cranial part named head-kidney, and the thymus. Secondary lymphoid organs, that is the centre of mature naive lymphocytes and the initial area of adaptive immune responses, include the spleen, the kidney (functions as both a primary and secondary lymphoid organ) and the lymphoid tissues present at various mucosal surfaces often termed as mucosa-associated lymphoid tissues (MALTs) [54]. It is worth mentioning that germinal centres as known from mammals, i.e. designated sites within secondary lymphoid organs where mature B cells proliferate, differentiate and mutate their antibody genes (somatic hypermutation), have so far not been reported in fish [39].

Immune cells in fish

Morphologically, the differences between fish and mammalian immune organs are prominent. However, when comparing immune cells and genes, the similarities are easily identifiable. Truly, cod (*Gadus morhua*) has been shown to lack the gene products of MHC class II and CD4 [55], but this exception in the organization of immune genes was very surprising. Rather, the key genes, transcripts and molecules of the adaptive immune system seem to be highly conserved throughout evolution from jawed fishes and onwards [39]. However, at the cellular level, certain important differences should be highlighted. These include functional differences such as the capacity of fish erythrocytes to form rosettes round antigens to trap antigens [56] and the capacity of B cells to act as macrophages [57, 58]. Nevertheless, the leukocytes in teleost fish largely follow the same classification as those in mammals. They include lymphocytes (T- and B cells), phagocytes (mononuclear cells, neutrophils and eosinophils) and auxiliary cells (basophils, mast cells, platelets). Some intriguing differences exist though. These include rodlet cells and melano-macrophages. Rodlet cells are hitherto only demonstrated in teleosts and are mainly present at mucosal surfaces. The cells have a characteristic structure with clublike inclusions. Their immune function and origin are uncertain, although present evidence points to a functional role in the host defence against parasites [59, 60]. The melano-macrophages are of special

interest in this thesis. They are thought to represent a sub-population of macrophages in ectothermic vertebrates which produce melanin [61]. In healthy salmon, they predominantly reside within some immune organs including the head-kidney and spleen [62], however, they can also be found in areas of chronic inflammation such as vaccine-induced peritonitis [5]. Melano-macrophages and their role in focal melanised changes are the focus of this thesis and a comprehensive background on these cells is given under 7.5.1.

Macrophages

The more common and amelanotic macrophages and their precursors (monocytes) are the main phagocytic cells of the immune system, which are rapidly recruited to the tissue upon injury or infection and differentiate into phagocytic macrophages capable to take up antigen and produce different cytokines [18]. The macrophages are known for their plasticity and can read cellular environmental changes to adapt their functional phenotype [63]. By simplified classification, macrophage phenotype has been divided into two groups: M1 (classically activated macrophages) and M2 (alternatively activated macrophages) [63]. Macrophages in fish largely resemble the phenotypes of mammalian macrophages, but in fish, a total of four different phenotypes are recognized: innate-, classic-, alternative- and regulatory macrophages [63]. Whereas innate macrophages are induced by pathogens (PAMPs) alone, classically activated macrophages are induced by a combination of interferon gamma (IFN-γ), produced mainly by Th1 lymphocytes, and a microbial stimulus [63]. However, both types of macrophages have an elevated phagocytic activity, expression of pro-inflammatory cytokine genes and produce reactive oxygen species (ROS) and nitrogen radicals (caused by upregulation of inducible NO synthase - iNOS). Alternatively activated macrophages are induced by a Th2 cytokine environment. In mammals, this occurs in the presence of IL-4 and/or IL-13. This activation pathway has not been recognized in teleost fish (reviewed in [63]). The alternatively activated macrophages of mammals metabolize L-arginine differently from innate/classically activated macrophages, by converting L-arginine into L-ornithine and urea through activation of arginase [64]. This attenuates the production of NO, as L-arginine is rendered unavailable for conversion by iNOS, and thus alternatively activated macrophages act like “anti-inflammatory” macrophages involved in repair and regeneration. Finally, regulatory macrophages are in mammals

differentiated in the presence of IL-10, which is involved in down-regulation of inflammation. IL-10 is also present in different teleost species; however, this activation pathway has not been determined in fish [63].

Lymphocytes

Cells of the lymphoid lineage include B and T lymphocytes and natural killer (NK) cells. The term "lymphocyte" was prompted because they are the dominant cell types of mammalian lymph. As the presence of lymphatic vessels is uncertain and structural lymph nodes are lacking in fish [42, 53], this term can be disputed. Nevertheless, B and T lymphocytes have retained this name in all jawed vertebrates where they mediate the specific immune responses of the adaptive immune system, equivalent to their function in mammals. NK cells seem somewhat left on the side-line in fish immune research. They are indeed cells of lymphoid origin; however, they utilize innate nonspecific receptors to recognize antigens and possess cytotoxic granules used in their killing activity [49]. This is in contrast to B and T cells, which have the unique process of somatic DNA rearrangements of antigen-specific receptors (B cell or T cell receptor – BCR or TCR) by random combination of the variable gene segments present in the receptor locus [38]. This results in a vast repertoire of B and T cells with structurally diverse antigen receptors able to react specifically with only one antigen [38]. As much focus has been directed towards B and T cells, the contribution of NK cells in fish immunity is largely left unexplored.

B cells express their antigen receptor on the cell surface known as BCR or as secreted proteins known as antibodies or immunoglobulins (Igs). Igs can specifically neutralize pathogens and label them for destruction by other cells of the immune system. Thus, antibody production is one of the major functions of B cells, in addition to antigen presentation and, in the case of teleosts, phagocytosis [57]. Phagocytic B cells have recently also been reported in reptile and mammalian B cells [65-68], however, teleost B cells are proposed to have a higher capacity for antigen presentation, which probably makes this property important in teleost B cells [69]. In Atlantic salmon, three different Igs have been reported: IgM, IgD and IgT, defined by the heavy chains μ, δ and τ, respectively [70]. To date, most studies have focused on IgM-bearing and IgM-secreting cells, as IgM is the major systemic antibody in teleost fish [70]. Less is known about IgD and IgT,

although studies in rainbow trout have indicated an anti-viral role for IgD in the gills [71], whereas IgT responses seem to dominate primarily in the gut [58].

T cells can both coordinate the responses of other immune cells or directly act as effectors to kill other cells [38]. They are, as in mammals, characterised by their TCR which they use to recognise antigens [49]. The TCR complex is in most cases restricted to recognizing antigens that are exposed on a major histocompatibility complex (MHC) (class I or II) on the surface of an antigen-presenting cell [72]. A broad classification based on TCR chains allows the differentiation between so-called αβ (alphabeta) and γδ (gammadelta) T cells [49]. αβ T cells are accounted as the conventional T cells, which again can be divided into T cytotoxic (Tc) or T helper (Th) cells, which differ according to the presence of either CD8 or CD4, respectively. CD8 and CD4 are two unique membrane-bound glycoproteins that act as co-receptors for the TCR and stabilizes the TCR/MHC interaction. CD4 T cells produce cytokines and regulate the actions of other immune cells, mainly B cells, and from mammalian immunology, the CD4 T cells can be further subdivided into different categories based on which cytokines they secrete and various specific transcription factors. Some well-defined subsets from mammals are Th1, Th2, Th17 and T reg [49]. It is speculated that fish have all the different T cell subsets as mammals as most of the components associated with T cell function have been identified genetically [73]. However, the functional properties of different subsets are subject for further investigation. CD8 T cells can directly kill infected or cancerous cells after recognizing foreign antigens presented on MHC class I, e.g., virus-infected target cells. The effector functions of CD8 T cells are related to the release of different cytotoxins such as perforin and granzymes, which enter the cytoplasm of the target cell and trigger the caspase cascade, eventually leading to apoptosis [38].

The γδ T cells can more easily be compared to innate-like immune cells. They are far less dependent on MHC presentation and can recognize unprocessed antigens in a similar fashion as the PRR-PAMP interaction [49]. They are strategically located in epithelial and mucosal tissues and their highest prevalence has been reported in the gut mucosa, within a population of leukocytes known as intraepithelial leukocytes (IELs) [72]. Still, the γδ T cells only account for around 2% of the total T cell population in mammals [74]. The functional role of γδ T cells in fish is yet to be investigated.

Soluble immune mediators

Different immune cell types secrete various soluble immune mediators, either constitutively or induced upon stimulation, in order to maintain tissue homeostasis [38]. Within these soluble mediators, the cytokines comprise a broad category of small proteins that regulate immune responses. Upon stimulation, leukocytes produce and release cytokines and regulate the immune responses accordingly through specific cytokine-receptor binding, either on the same cell (autocrine) or other cells (paracrine). Further, the cytokines related to particular immune functions can be sub-grouped into chemokines, interleukins, interferons (IFNs) and tumour necrosis factors (TNFs), among others [49]. While chemokines are produced as "chemo-attractant molecules", i.e. to attract cells to sites of infection/inflammation, interleukins act more as signalling molecules between leukocytes [75]. Their functions are plentiful and highly variable depending on the cell source and the target cells. IFNs are so-called virus-inducible cytokines with strong antiviral activity [76]. TNFs have also been attributed a vital role in the antiviral defence [77].

Immune responses to viral infection with emphasis on PRV-1

Both the innate and the adaptive immune systems respond to viral infections and are crucial for the anti-viral defence. Depending on the virus and route of entry, some viruses infect epithelial cells and others pass the epithelial barriers through infection of immune cells and may enter the blood stream inter- or intracellularly to seek predilection organs or tissue and replication. The innate immune system recognizes viral PAMPs. The PAMPs that recognise viruses are typically located on endosomal membranes, because endocytosis is a common way of entry of virus from the extracellular space to the cell's interior. The PAMP recognise exposed virus-specific molecules. These can be dsRNA, ssRNA with 5' and 3' ends differing from cellular produced RNA or DNA at the "wrong" place in the cell. The PRRs known to be important in dsRNA detection include RLRs, TLR3 and TLR22 [78]. Upon viral detection and PRR/PAMP interaction, the cells initiate transcription of IFNs and genes of pro-inflammatory cytokines [59].

dsRNA is a strong alarm signal for the innate immune response, and therefore an especially potent trigger of innate immunity. Furthermore, dsRNA is not naturally present in cells and is thus interpreted as a virus specific antigen. PRV-1 has a dsRNA genome, which is boxed in

a capsid of two layers of protein. However, in an ideal replication cycle of reoviruses, including PRV-1, the dsRNA is never exposed to the cell. The virus is engulfed by endosomes and only the outer layer of the capsid is removed through the virus' ingress to the cytoplasm. The inner layer, or the "core particle", resides in cytoplasm and is not decomposed and expels newly synthesized viral mRNA through windows in the core particle. The viral mRNAs direct the cell to produce viral proteins, these proteins are used for making new viral particles, and new viral genomic RNA are encapsidated as ssRNA before dsRNA are produced. In this way, the viral dsRNA is never exposed to the cell. Indeed, in PRV-1 infection of Chinook salmon (a Pacific salmon species) the virus replicates, but the innate or adaptive responses, if any, are below detection limit, which indicate a lack of stimulation of the innate responses [79].

In PRV-1 infection in Atlantic salmon, on the other hand, virus responsive genes are induced, including interferon-regulated anti-viral genes, as well as genes involved in antigen presentation via MHC class I [80, 81]. The anti-viral genes have been shown to stay upregulated for at least ten weeks after primary PRV-1 infection [82]. This coincides with stimulation of negative regulators suppressing other immune pathways and proinflammatory responses, such as induction of suppressor of cytokine signalling 1 (socs1) and IL-10 receptor (il10r) [80]. The following adaptive immune response, and the cellular (cytotoxic) response, is important in the anti-viral defence of salmonids. A T cell mediated response has been shown in ISAV, SAV and PRV-1 infection [74, 83-87], and for HSMI, a cytotoxic $CD8^+$ myocarditis has been shown [85]. On the genetic level, expression of genes associated with differentiation and maturation of B lymphocytes and cytotoxic T cells has been shown to be increased [81]. Melanin accumulation in heart or in white muscle has never been reported as a common finding in HSMI. On the other hand, pathological pigmentation in the cardiac tissue of fish with CMS has been shown [88], but this is not a common finding.

7.5 MELANIN AND THE PIGMENTARY SYSTEM

7.5.1 What is melanin?

Melanin comes from the Greek word *melanos*, meaning "dark". The term was probably first used by the Swedish chemist Berzelius in 1840 in naming a dark pigment extracted from eye membranes [89]. Melanin is frequently considered just an animal cutaneous pigment serving as

photo-protection against UV radiation [90]. However, there is more to melanin than that. Melanin is merely the generic name used to refer to perhaps the most ubiquitous, resistant, heterogeneous and ancient polymer found in nature [91]. This complex group of pigments are produced in all phyla, including microbes, insects, plants and primates to name a few. A wide definition including all types of melanin would be "*a heterogeneous polymer derived by the oxidation of phenols and subsequent polymerization of intermediate phenols and their resulting quinones*" [91]. Thus, their structure is relatively diverse and undefined. Melanin can be further classified in at least five main types according to the source: animal melanin, plant melanin, fungal melanin, bacterial melanin, and synthetic melanin [91].

There are two major types of animal melanin, both made in specialised organelles in melanin-producing cells called melanosomes, and these are termed eumelanin and pheomelanin [92]. Eumelanin is brown-black in colour and pheomelanin tends towards yellow/red/brown. These two dominant types of melanin differ in composition and thus also in chemical and physical properties. While eumelanin is stable and largely insoluble, pheomelanin is alkali-soluble [93, 94]. The biosynthetic pathways to eumelanin and pheomelanin begin with the amino acid tyrosine and the key enzyme tyrosinase. Further activities of the enzymes Tyrosinase related protein-2 (Tyrp-2) and Tyrosinase related protein-1 (Tyrp-1) are also necessary. In the production of pheomelanin, only the presence of tyrosinase (and the presence of cysteine) is needed.

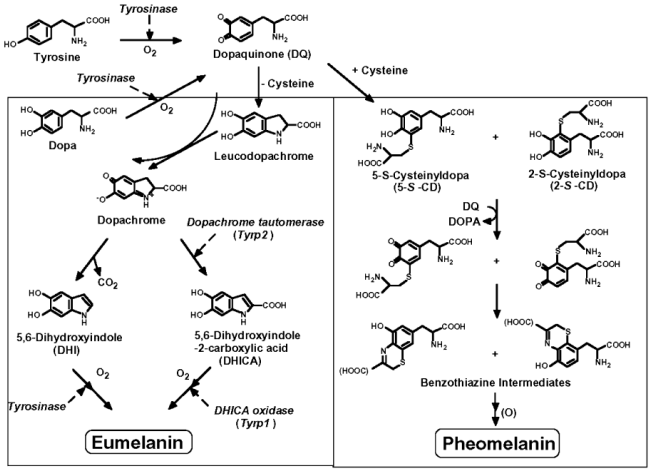

Figure 6. The biosynthetic pathway to eumelanin and pheomelanin. Note that only tyrosinase activity, in the presence of cysteine, is necessary in the production of pheomelanin (derived from Wakamatsu et al. 2002 [95] with permission from Elsevier Ltd.).

7.5.2 Melanin-producing cells

Melanocytes of mammals and birds

When addressing the pigmentary system of higher, homoeothermic vertebrates (i.e., birds and mammals), focus is on the melanocyte, the cell type responsible for melanin production. The major proportion of classic melanocytes originate from the neural crest (ectoderm) and migrate mainly to the skin and mucous membranes [93]. Here, melanin is synthesized in subcellular organelles called melanosomes [96]. This process is termed melanogenesis and is driven by specific enzymes encoded by genes of the tyrosinase gene family (**Figure 6**) [95]. Arguably the best-known property of melanin is its ability to absorb and reflect light, thus protecting epidermal and dermal cells from UV radiation [93, 97]. After melanosomes are shredded from melanocytes, they are internalised by keratinocytes where they create a protective shield overlying the cell nucleus [98]. The uptake of melanosomes in keratinocytes is also the reason for differences in external pigmentation. However, the presence of melanin at other extra-cutaneous sites suggests other additional functions. Melanocytes can be found in the inner ear, in the choroid of the eye and in the meninges, where melanin is thought to absorb and neutralize free radicals and

bind toxic compounds such as heavy metals and organic cations [93]. Also, melanin can be encountered in the central nervous system (neuromelanin) where it is synthesised by the neural cells themselves [99]. Neuromelanin is especially abundant in the *substantia nigra* of the midbrain and thought to protect neural cells from iron-induced oxidative stress [100]. Importantly, the melanocytes of mammalians must not be confused with melanophages. Melanophages are not able to synthesise melanin and are merely thought to phagocytose melanin, e.g. from lysed melanocytes/keratinocytes [101].

Teleost melano-macrophages

In fish, there are many different pigment cell types [102]. The melanin-containing cells in the fish skin are termed melanophores, mainly present in the dermis where they produce black pigments. Other pigment cell types in fish include the erythrophores (red or yellow) and iridiphores (whitish), also derived from the neural crest [102]. In contrast to higher vertebrates, ectothermic vertebrates also possess a well-developed extracutaneous pigmentary system (also known as visceral or internal pigmentary system) with melanin-containing cells termed melano-macrophages [95]. The melano-macrophages are thought to be of the myeloid cell lineage and should not be confused with other pigment-producing cells of fish that are derived from the ectoderm. The cells first appear within haematopoietic tissues at a time following first feeding, which correlates well with the appearance of immunological maturity [103]. In many ectothermic vertebrates, the melano-macrophages are aggregated in so-called melano-macrophage centres close to vessels in their housing organs. This is not true in salmonids, where the melano-macrophages are seemingly distributed randomly in the tissue stroma, but still close to vessels and other immune cells [104, 105]. The melano-macrophage centres have on both functional and structural grounds been suggested to represent the primitive analogues of germinal centres of lymph nodes of mammals and birds [46-48, 104]. The centres also act as dumping sites, where destruction, detoxification or recycling of different endogenous and exogenous materials occur [104]. Free radicals from degraded phagocytised material can be neutralised by melanin, thus acting as an antioxidant [104].

The melano-macrophages are capable of melanin synthesis [62] and are thought to be part of the mononuclear phagocyte system normally resident in the spleen and kidney. They are normally located side-by-side with various haematopoietic cells and can trap antigens and create an appropriate immune response. As with other professional antigen-presenting cells, the melano-macrophages may express MHC class II which can further stimulate and attract T cells [106]. In relation to vaccination, the melano-macrophages have been shown to retain antigens for a prolonged time period after vaccination, thus creating a depot effect or persisting antigen presentation [105, 107]. The cells can thus not only react in lymphoid organs, but also be recruited to an inflammatory site, e.g., the peritoneum after vaccination. This is especially true for chronic and granulomatous inflammation, where the activity of melano-macrophages often is upregulated [5, 108]. Although much information regarding both distribution and function of melano-macrophages is at hand, the relation between the pigmentary and immune system in teleosts is still poorly understood.

7.6 MELANISED FOCAL CHANGES IN WHITE MUSCLE

7.6.1 Naming of the condition

Melanised focal changes have been termed black spots or melanised spots in previous papers and reports. In this thesis, they are referred to as *melanised focal changes*. This complies with proper pathological description and terminology, including the extent and the nature of the pigmentation of the changes. It is important to specify these changes as focal, as there are other manifestations that are more diffuse or solely present in the red musculature. The naming also provides the opportunity to differentiate between melanised and haemorrhagic changes, the latter being termed *red focal changes*. Additionally, *black spots* should be avoided when describing this condition, as *black spot disease* is a freshwater fish disease caused by flatworm larvae of the genus *Neascus* [109]. Similar naming of these very different conditions could be confusing.

7.6.2 Melanised focal changes: A quality problem or a disease?

Quality can be defined as *the standard of something as measured against other things of a similar kind* [110]. Salmon fillets are graded prior to sale according to their quality. The grading system is divided in three categories: production (low), ordinary (medium) or superior (high). The grade

is determined by several factors, including erroneous filleting, scale loss, sexual maturation, fin damage, deviant pigmentation/colouration, wounds, deformities and bleedings or melanin in the filet. Any form of melanisation of the filet impairs product quality and the filet is consequently graded as production fish. As such, melanisation is regarded as a quality problem and not a disease by the fish farming industry. However, this is debatable.

Disease can be defined as *a disorder of structure or function in a human, animal, or plant, especially one that produces specific symptoms or that affects a specific location and is not simply a direct result of physical injury* [110]. Melanised focal changes are indeed a disorder of structure and function. Symptoms of the condition have not been identified, but considering the tissue damage, muscular pain is probable. Attempts to measure plasma enzymes such as creatin kinase (CK) have failed to show correlation in field trials [4]. The changes occur predominantly in the cranio-ventral region of the fillet, i.e. a specific location within the muscle tissue [4]. The chronic nature of the changes argues against traumatic injury, and for a non-resolving condition, as a muscular injury would be expected to heal fully. In addition, the changes are mostly located in the depth of the fillet towards the peritoneal side, and not towards the skin. As such, melanised focal changes can also be regarded as a muscle disease. Thus, melanised focal changes as a condition are found at the intersection between a quality problem and a muscle disease with overlapping characteristics fitting both definitions. While the quality aspect is suitable in a commercial context, the disease aspect would provide the proper terminology to be used in scientific investigations addressing the pathological and immunological responses within such changes.

7.6.3 Aetiology – one or multiple causes?

The first report on melanised focal changes in fillets of farmed salmon appearing in a peer-reviewed journal was published in 2005 [5]. The condition was described as pigmented foci occurring as a result of chronic granulomatous inflammation and melanin accumulation [5]. At this time, the hypothesized aetiology was intraperitoneal vaccination. Oil-based vaccines injected into the peritoneal cavity were thought to induce inflammatory reactions affecting the peritoneum and the peritoneal wall but occasionally also the underlying white muscle, causing chronic myositis with the potential to develop into melanised foci. Vaccination was a plausible

cause for several reasons: melanised foci have never been registered in wild salmon (if found, these were determined to be escaped farmed salmon), which are unvaccinated. The vaccines used for farmed salmon are based on non-metabolizable mineral oils, which induce strong immune reactions of granulomatous character and the side effects of using such vaccines in salmon have been thoroughly investigated [111, 112]. Finally, fat droplets in the lesions were interpreted as adjuvant oil accumulations promoting inflammation. However, later in two independent reports, non-vaccinated farmed salmon were also found to develop focal melanised changes, disproving the hypothesis that the aetiology was solely related to vaccination [113, 114]. Nevertheless, we do know that erroneous injection of vaccine can result in local melanisation and this is probably causative in some cases. In retrospect, the study would have benefited from a classification system based on more than just a registration of the occurrence of melanised lesions, assuming them all to be manifestations of inflammation with identical aetiology.

Although the first (peer-reviewed) report on focal melanised changes in farmed salmon was published in 2005 [5], such changes have been known to occur prior to this time, though at a very low prevalence. Historical data from Mowi show a gradual increase in prevalence from the early 2000's until today (personal communication, Øyvind Oaland, Mowi). Interestingly, the first report on HSMI also dates back to the early 2000's [25], and a similar development with increasingly more breakouts of HSMI correlates with the increased occurrence of melanised focal changes.

The effects of other production related factors, such as ploidy, smolt production regime and different diets, have also been addressed [4, 113, 115]. In one study, there were more fish with melanin deposits amongst triploids compared with their diploid counterparts [113]. In the same study, smoltification at elevated temperature after vaccination increased the number of affected individuals compared with vaccination followed by simulated natural smoltification [113]. Although these factors affected the frequency of melanin, all changes appeared similar at the histological level, and none of these factors alone was thought to be imperative for the pathogenesis of the condition.

Effects of various feed components and different inclusions of certain ingredients have been investigated in several feed trials, with the common conclusion that elevated levels of the essential omega-3 fatty acids EPA and DHA result in a lower prevalence of focal melanised changes. Most of these investigations have been conducted by the feed companies themselves and the results are not available in referrable publications. Interestingly, a feed trial where fish with a PRV-1 and SAV3 co-infection were given a leaner, more protein-rich feed (25% fat/46% protein), led to increased survival and significantly less focal melanised changes as compared with fish in the control group given an ordinary growth feed (35% fat/37% protein) [115]. This is most likely due to the fatty acid profile of standard growth feeds, where plant oils (mainly rapeseed/colza oil) are the dominant source that have an omega-6:omega-3 ratio skewed towards omega-6 (3:1). The pro-inflammatory properties of omega-6 is probably important for the development of focal melanised changes. Although the prevalence of changes can be manipulated by pro- and anti-inflammatory feed ingredients, the feed is not considered as the cause of the condition. Another similar effect can be attributed to the level of antioxidants, where an increase in antioxidants can decrease the frequency and the severity of the changes [4]. Addition of zinc, which is important in wound healing, has also been shown to have a positive effect [4].

Of other unpublished data, several fish farming companies report that fish in small sea cages have less melanised changes at slaughter (personal conversation with industry representatives). Also, ecologically farmed fish are reported with lower prevalence melanised changes at slaughter. This could be due to changes in feed composition (increased fish oil), however, production of ecologically farmed salmon also demands less crowded conditions in the sea cages with only 10kg per/m^3 (25kg per/m^3 in standard production). This can provide better conditions in terms of water quality and oxygen levels. In a controlled experiment, lowering of the oxygen level for a short period of time 2 months prior to slaughter resulted in an increased prevalence of focal melanised changes [4]. Notably, oxygen levels have recently received special attention in Chile, where much of the production facilities are located to a restricted geographical area where oxygen levels have been a recurrent problem. This has coincided with increased prevalence of focal melanised changes, or “melanosis” as it is commonly referred to in Chile. In

this context, it is also worth mentioning that PRV-1 infection and HSMI is a fairly new diagnosis and emerging disease in Chile, with the first report published in 2016 [116].

In summary, factors such as smoltification, ploidy, production scale and feed can interfere with the frequency of changes and to a certain extent with the severity of the changes, however, none of them seems to be directly causative. Infectious diseases, however, remain somewhat unexplored. Investigations targeting bacteria have been unsuccessful and the outcome of viral infections is largely unknown. It has been reported that the prevalence of melanisation in general (not only focal changes) increases after PD (both SAV3 and SAV2 infection) [4], however, these results remain somewhat anecdotal. Larsen et al. [108] did investigate focal melanised changes for the presence of SAV, IPNV and PRV-1, but without any findings. In addition, Fagerland et al. [88] reported melanisation of the heart in relation to CMS, but this was not put into context with focal melanised changes. Krasnov et al. [117] detected prokaryotic rRNA by RNA-seq analysis with preferential or exclusive location to focal melanised changes, suggesting a bacterial component to the pathogenesis. Microbial contamination during sampling was not ruled out in this context.

7.6.4 The inflammatory milieu in melanised focal changes

Melanised focal changes have been characterized both histologically and by transcription analysis in several studies [4, 108, 117]. Histological investigations have revealed muscle degeneration and necrosis, fibrosis and granulomatous inflammation containing varying numbers of melano-macrophages. The granulomatous inflammation is analogous to the pattern found in mammals, with abundant MHC class II^+ cells and scattered T cells. Well-organised granuloma may be detected within the changes, although the condition seems heterogenous, with diffuse granulomatous inflammation occurring in some changes, or in parts of the changes. The condition was thus termed a polyphasic necrotising myopathy, without any further histological classification of changes. A polyphasic myopathy points towards a stimulus over a longer period, resulting in muscle cells in different phases of degeneration and regeneration. This contrasts with a monophasic myopathy where most affected cells are in the same phase of either degeneration or regeneration, and where the stimulus is of short duration, e.g., a traumatic event.

Transcriptional analysis using RT-qPCR of pigmented samples has shown an upregulation of MHC class II and mIgM genes [108]. This was further supported by transcriptome profiling, revealing massive up-regulation of MHC class II, helper T cells and B cells, including induction of immunoglobulins [117]. Genes associated with melanogenesis have also been shown to be upregulated [117] and the presence of transcripts of key enzymes in melanogenesis has suggested *de novo* synthesis of melanin in melanised focal changes [108].

7.7 KNOWLEDGE GAPS

- The possible link between red and melanised focal changes
- The involvement of infectious agents in the development of melanised focal changes
- The development and prevalence of focal melanised changes during the seawater phase
- A model for induction of focal melanised changes to study the progression of the condition
- The inflammatory milieu in focal red and melanised changes

8 AIMS OF THE STUDY

The main goal of this study was to reveal the pathogenesis of "black spots" (melanised focal changes) in Atlantic salmon white muscle, with emphasis on understanding the development of the condition. As *Piscine orthoreovirus* 1 (PRV-1) is known to cause heart and skeletal muscle inflammation (HSMI) and thus is related to other disorders of the musculature, an important first objective was to investigate the potential presence of PRV-1 in melanised focal changes. Thus, a primary hypothesis was postulated: PRV-1 is present in melanised focal changes. The outcome of this hypothesis would determine subsequent investigations. To test this hypothesis, the following sub-goal was defined:

1. To determine the presence of PRV-1 in melanised focal changes using both protein and gene expression analysis (Paper I).

As PRV-1 indeed was detected in melanised focal changes following this initial investigation (Paper I), the subsequent goal thus became to investigate the potential role of PRV-1 infection in the condition. Also, industry representatives had reported on a modest occurrence of "red spots" (red focal changes) in the same anatomical location of the filet as melanised focal changes, and

these were presumed to be an initial manifestation of the condition. Potentially, these changes might occur because of PRV-1 infection and this need to be tested.

To fulfil the research goal, the following hypothesis was postulated: PRV-1 infection causes acute red focal changes in the musculature, which can progress into a chronic inflammatory condition with the local presence and accumulation of melano-macrophages, thus developing into melanised focal changes. To test the hypothesis, the following sub goals were defined:

1. To determine the presence of PRV-1 in red focal changes using both protein and gene expression analysis (Paper I and II).
2. To characterise red focal changes macroscopically and microscopically (Paper I and II).
3. To reveal key features of red and melanised focal changes and investigate whether the changes are different (time-related) manifestations of the same condition or not (Paper I and II).
4. To investigate the frequency and development of red and melanised focal changes in relation to the onset of PRV-1 infection in the seawater phase of farmed salmon (Paper III).
5. To induce experimentally red and melanised focal changes in Atlantic salmon in a challenge study with PRV-1 and study their development (Paper III).
6. To reveal possible inflammatory responses in red focal changes from slaughter size fish (PRV-1 positive fish), and if present, characterize and compare them with the inflammatory responses in melanised focal changes (Paper I and III).
7. To investigate the potential role of macrophage polarity and the cell mediated immune response with regard to the development of red and melanised focal changes in both PRV-1 infected and non-infected fish (Paper IV).

9 SUMMARY OF SEPARATE PAPERS

Paper I:

Melanised focal changes (black spots) are common findings in the white skeletal muscle of seawater-farmed Atlantic salmon (*Salmo salar*). Fillets with melanised focal changes are considered to be of lower quality and are the cause of large economic losses. It has been suggested that red focal changes (red spots) precede the melanised focal changes. In the present work, different populations of captive and wild salmon were examined for the occurrence of both types of changes, which were investigated for the presence of different viruses by immunohistochemistry and RT-qPCR. The occurrence of red or melanised foci varied significantly between the populations, from none in wild fish control group, low prevalence of small foci in fish kept in in-house tanks, to high prevalence of large foci in farm-raised salmon. Large amounts of *Piscine orthoreovirus* (PRV-1) antigen were detected in all foci. No other viruses were detected. Red focal changes contained significantly higher levels of PRV-1 RNA than apparently non-affected areas in white muscle of the same individuals. Some changes displayed a transient form between a red and melanised pathotype, indicating a progression from an acute to a chronic manifestation. It was concluded that PRV-1 is associated with the focal pathological changes in the white muscle of farmed Atlantic salmon and is a premise for the development of focal melanised changes.

Paper II:

Melanised focal changes in skeletal muscle of farmed Atlantic salmon (*Salmo salar*) are a major quality problem. The aetiology is unknown, but infection with *Piscine orthoreovirus* (PRV-1) has been associated with the condition. Here, the pathogenesis of red and melanised focal changes and their association with PRV-1 was addressed. First, a population of farmed fish (PRV-1-negative prior to sea transfer) was sequentially investigated throughout the seawater period. The fish were autopsied and tested for PRV-1 infection. Muscular changes were described by macroscopy and histology, and a classification system was established. Second, in an experimental infection trial, PRV-1 was injected intramuscularly to induce changes. The farmed fish was gradually infected with PRV-1. Red focal changes occurred throughout the observation

period with a low prevalence regardless of PRV-1 status. Melanised changes were highly diverse, and their prevalence increased during the trial. Changes of low macroscopic grade and histological category were more prevalent in PRV-1-negative fish. Diffuse granulomatous melanised changes only occurred after PRV-1 infection. No muscular changes were observed in the experimentally challenged fish. These studies do not indicate that PRV-1 infection causes red focal changes but seems important in the development of granulomatous melanised changes.

Paper III:

Farmed Atlantic salmon (*Salmo salar*) are prone to various conditions affecting the quality of the fillet. A well-known but so far poorly understood condition is the focal red changes in muscle, often referred to as haemorrhages. Such changes are characterized by muscle necrosis, haemorrhages and acute inflammation. They can progress into focal melanised changes, which is a chronic inflammatory condition with melanin-producing leukocytes. The initial cause of intramuscular haemorrhages is unknown. This study aimed to reveal some of the key immunological features. Samples of red focal changes were investigated by immunohistochemistry (IHC), *in situ* hybridization (ISH) and RT-qPCR for various immune markers. ISH targeting 16S rRNA was also performed to address the potential presence of bacteria. The results were compared with samples of melanised changes and control muscle, subjected to the same analyses. In all red changes, infiltrates with mononuclear cells were detected, consisting mostly of MHC class I/II+ cells, but also of CD3+ and CD8+ cells. ISH studies on IgM showed few to moderate amounts of B-cells in red focal changes. Trends in the RT-qPCR showed upregulation of genes related to innate immunity in the red changes, whereas genes related to adaptive immunity were upregulated in the melanised changes. An important result was the significant downregulation of the anti-inflammatory cytokine IL10 in all red changes. These findings indicate that an auto invasive nature of the changes can be ruled out. The downregulation of IL10 at an early phase was shown to be a trait for the condition. Most red and melanised changes did not show labelling for 16S rRNA. Scattered signal was detected in some changes, but this was not in relation to pathological changes and mostly in the periphery of the tissue, possibly due to bacterial contamination during handling of the samples.

Paper IV:

Melanised focal changes in white skeletal muscle of farmed Atlantic salmon, “black spots”, is a quality problem that affects on average 20% of slaughtered fish. The spots appear initially as “red spots” characterized by haemorrhages and acute inflammation and progress into black spots characterized by chronic inflammation and abundant pigmented cells. *Piscine orthoreovirus* 1 (PRV-1) has earlier been found to be associated with macrophages and melano-macrophages in red and black spots.

In this study, the inflammatory microenvironment of red and black spots was addressed by studying the polarization status of the macrophages and cell mediated immune responses, in both PRV-1 infected and non-infected fish. Samples that had been collected at regular intervals through the seawater production period in a commercial farm were analysed by multiplex fluorescent *in situ* hybridization (FISH) and qPCR methods. Detection of abundant inducible nitric oxide synthase (iNOS2) expressing M1-polarized macrophages in red spots demonstrated a pro-inflammatory microenvironment. There was a high level of colocalization between the iNOS2 expression and PRV-1 infection. Black spots, on the other side, had few iNOS2 expressing cells, but a relatively high number of arginase-2 expressing anti-inflammatory M2-polarized macrophages containing melanin. The numerous M2-polarized melano-macrophages in black spots indicate an ongoing healing phase. Colocalization of PRV-1 and cells expressing $CD8^{+}$ and MHC-I suggests a targeted immune response taking place in the spots. The findings of this study indicate that PRV-1 induces a pro-inflammatory environment that is important for the pathogenesis of the spots. There was no indication that infection of PRV-1 is the initial causative agent of this condition.

10 DISCUSSION

10.1 MATERIAL

In this thesis, samples from Atlantic salmon from various origins were investigated for the presence of focal red and/or melanised changes. Handling, anaesthesia and euthanasia were performed according to national guidelines (Akvakluturforskriften, §34. Avlivning av fisk). In Paper I, both farmed and wild salmon and salmon from experimental in-house tanks were included, both vaccinated and un-vaccinated. Parts of this material were also used in Paper III. Farmed fish were obtained at the abattoirs from different parts of Norway and the wild fish were caught in the river Drammenselven in Buskerud, Norway. As PRV-1 is ubiquitous in farmed salmon in the seawater phase along the entire southern, western and northern coast of Norway, we collected wild fish from the eastern part of Norway, far away from fish farming sites. The wild fish proved negative for PRV-1 and no muscular changes were detected. Thus, this fish served as a control group. Importantly, the wild fish were caught upriver and hence living in fresh water where they go for spawning. During this period, the fish also stop eating. These processes imply major physiological changes. Whether or not this has an impact on the defence against PRV-1 and the development of muscular changes is unknown.

In Paper II, farmed fish from one production site in the Hardangerfjord were followed by sequential samplings from the beginning to the end of the production period. A field trial was the only option to investigate the development of muscular changes and the PRV-1 status over time, as there is currently no way of inducing such changes under controlled experimental settings. The advantage of a field trial is that the results are highly valid for the fish farming industry. The disadvantage of a field trial is the loss of control of external factors. The influence of environmental variations and other infectious agents than PRV-1 could have affected the results. The effects of a co-infection with PRV-1 and SAV3 have been speculated upon [37], as outbreaks of PD close to slaughter have been reported to increase the prevalence of large, melanised changes in the fillet [38]. However, there is no evidence that SAV3 has a causative role in the development of focal red and melanised changes. Importantly, SAV3 is only endemic on the western coast from Rogaland in the south to Møre and Romsdal in the north, while focal melanised changes occur along the entire coast.

In addition, cultivated fish were used in an infection trial aiming to induce muscular changes (Paper II). The experiment was conducted in parallel to the field trial. After examining the results from the field trial, it became apparent that PRV-1 is not the initial cause of red focal changes, but only seems to affect the severity of melanised focal changes. With this in mind the experimental setup was not optimal for induction of red focal changes.

In Papers III and IV, material from Paper I and II was reused in the respective papers, both addressing the immune responses in red and melanised focal changes. The material was fixed (at least 24 h, but no more than 48 h in formalin) and optimised for IHC and ISH.

10.2 METHODOLOGICAL CONSIDERATIONS

The methods used in the studies of this thesis include various morphologic and genetic techniques. Samples were fixed in either formalin or RNAlater and used in different analyses.

10.2.1 Histology

The purpose of the general histological investigations was to investigate the pathological features of the changes and possible presence of bacteria. The formalin fixed samples were paraffin-embedded, sectioned and stained according to standard protocols [39]. The stains used include hematoxylin and eosin (HE), Van Gieson's (to detect collagen) and Berlin blue (to detect iron). To investigate the possible presence of microorganisms, Gram, Giemsa, PAS and Ziehl–Neelsen stainings were performed. These methods are all well-established and common in histopathological diagnostic work.

10.2.2 Immunohistochemistry

Immunohistochemistry (IHC) is a powerful investigative tool in the assessment of pathological changes. The technique is based on the interaction between specific antibodies and target epitopes in the tissue. After antibody-antigen binding, the antibody can be visualized with a reporter system that produces colour. The colour reaction visualizes the distribution of the target epitopes within the tissue, providing valuable morphological information.

Formalin-fixed paraffin-embedded (FFPE) tissue is easy to work with and gives superior morphology as compared with frozen tissue and was thus the preferred way to sample material for the studies of this thesis. Another advantage of FFPE tissue is that, if fixed properly, the tissue

is also suitable for novel ISH techniques (more on ISH under 10.2.3). The disadvantage of such material is that formalin causes cross-links between adjacent proteins, which can mask antigens and interfere with the antibody-antigen binding, making IHC difficult and less sensitive. A necessary step in the protocol is antigen retrieval, which was achieved in Paper I-III by autoclaving of the sections.

The history of IHC has been a constant effort to improve the sensitivity of the methods. This has resulted new technological solutions, which are modifications and improvements of the initial method from the late 60's [40]. The IHC in all papers was performed using EnVision™+ System, HRP, DAKO, Glostrup, Denmark [41]. These kits are based on a two-step visualization protocol, where the primary antibodies bind to secondary antibodies with either anti-mouse Ig or anti-rabbit Ig specificity. The secondary antibodies are conjugated to an HRP-labelled (horseradish peroxidase) polymer. The secondary antibodies are universal and can be used to detect any primary antibody produced in either rabbit or mouse. Together, the primary and secondary antibodies form the two-step procedure, with the benefit of signal amplification, but also with the possibility of introducing non-specific labelling by the secondary antibody. To counter this, a blocking solution with normal serum from goat was used to block the secondary antibody from non-specific staining.

The labelled polymer conjugated to the secondary antibody does not contain (strept)avidin or biotin, and consequently, non-specific staining resulting from endogenous biotin in tissues such as liver and kidney is eliminated or significantly reduced, which increases the sensitivity and specificity of the method. However, the interpretation of the staining should be complemented by morphological investigations of the staining distribution and with proper positive and negative controls. Such were always included in all IHC runs performed, suitable for the antibody in question. E.g., for PRV-1 antibodies, control material from an infection trial with known Ct values was used.

To evoke colour, a suitable substrate-chromogen was added after incubation with the secondary antibody. When using the DAKO kits, there is a choice between two substrates: 3,3'-Diaminobenzidine (DAB) giving a brown colour precipitate or 3-Amino-9-Ethylcarbazole (AEC)

giving a strong red colour. DAB is more sensitive and gives cleaner background than AEC; however, AEC is less chemically hazardous than DAB. For the studies in this thesis, red staining was often preferred, as melano-macrophages can appear brown, varying according to the content of different pigments, and can easily be confused with immuno-positive (brown coloured) cells.

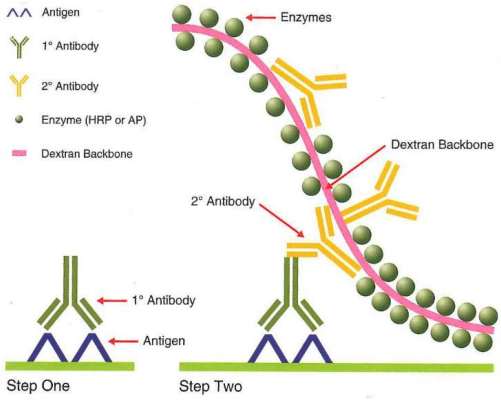

Figure 7. Schematic drawing of immunohistochemistry with EnVision+, HRP, by DAKO [41].

IHC was performed in Paper I-III. There were several purposes in using IHC. First, to detect viral proteins, i.e., presence of PRV-1 in melanised focal changes (Paper I-III). Second, to detect various immune cells in both red and melanised focal changes (Paper III). Only non-commercial primary antibodies were used. The antibodies targeting PRV-1 proteins have been thoroughly characterized in previous papers, validating their specificity and sensitivity [22, 23]. For validation of the antibodies targeting different immune molecules (Paper III), the reader is referred to the references given in the paper.

10.2.3 *In situ* hybridization

While IHC localises proteins in tissue sections, *in situ* hybridization (ISH) is a powerful hybridization technique that uses modified nucleic acid strands (probes) or labelled complementary DNA or RNA to localize specific DNA/RNA sequences in a tissue (thus, *in situ*). This is crucial for understanding the organisation, regulation and function of genes, while

simultaneously keeping spatial information. The current key ISH technique is based on hybridization to mRNA with RNA probes that can be visualised using a reporter system. The sections can be further analysed with light or fluorescent microscope, whichever is applicable, and mRNA can be localised and quantified within the sections.

The method of choice for the studies in this thesis was the novel *in situ* hybridization technique RNAscope© by Advanced Cell Diagnostics (ACD), BioTechne, California, USA. This is unique hybridization technique based on a novel probe design, which significantly diminished the chance of cross-hybridization. Reports on the method argue for high specificity, but also high sensitivity, as merely one RNA transcript in the tissue is enough for hybridization and visualization to occur. Briefly, the probes are constructed of up to 20 double Z target probe pairs, which are designed to hybridize specifically to the target RNA sequence. Both Z probes in a pair are required to bind for hybridization and further amplification to occur. This is the crucial step assuring high specificity. It is the lower part of the Z that is complementary to the target RNA, while the upper part of the Z has a sequence-binding region for preamplifiers. The preamplifiers are binding regions of amplifiers where fluorescence probe or chromogenic enzymes can bind, thus evoking a detectable signal. The method demands high RNA quality in the samples. Although RNAscope© is optimized for formalin-fixed and paraffin-embedded samples, fixation should not over exceed seven days. The material used in the included papers was never fixed for more than 48 hours.

The purpose of ISH was mainly to investigate the presence of bacteria (Paper III), virus (Paper III and IV) and to investigate macrophage heterogeneity (Paper IV) in red and melanised focal changes. Bacterial ribosomal RNA in melanised focal changes detected by RNA sequencing has been published by other researchers [42], however, this result has never been validated by other approaches. On the contrary, several studies have used conventional histological techniques to look for bacteria, but without results [4]. By ISH, bacterial transcripts can be visualized and seen in context with pathological changes and further our understanding of the condition. To investigate bacteria by ISH, a universal bacterial probe was designed targeting conserved 16S rRNA transcripts. 16S rRNA is a large gene present in almost all bacteria and the function of the gene over time has not changed, making it a common housekeeping genetic marker used in sequencing studies investigating bacterial phylogeny and taxonomy [43].

Although ISH targeting 16S rRNA transcripts are highly useful in detection of bacteria, it cannot discriminate between different bacterial species and only answer to the binomial question; are bacteria present in red or melanised focal changes or not.

ISH was also used to address the occurrence and distribution of IgM transcripts in Paper III. Although there are several antibodies working in IHC targeting Igs and IgM [118], ISH was preferred due to the sensitivity of the method. The probe was designed to target both membrane-bound and secretory IgM. The limited selection of salmon-specific antibodies is a general problem; however, this can be partially circumvented by using ISH. RNAscope© is already highly appreciated in other species where the lack of commercial antibodies is a problem.

An important issue for molecular biology is to establish whether transcript levels of a given gene can be used as proxies for the corresponding protein levels. Studies in other species than salmon show that the RNA-to-protein conversion varies a lot depending on the gene in question [119]. For example, expression data on Igs seem to correlate well with IHC results. This is also true for the genes of the melanogenesis pathway, where the expression fits well with the histological appearance of melanin-producing cells [62]. However, in the particular case of PRV-1, this discussion is especially interesting. PRV-1 infection is characterised by an early acute phase with high viral loads in blood [26]. At this stage, the virus replicates in its major target cell; the erythrocyte [21], and until peak infection, both PRV-1 RNA and protein levels increase in red blood cells [26]. Post peak infection, the viral protein levels drop substantially while RNA levels remain elevated [120]. The viral RNA after the peak phase, i.e., in the persistent phase is mainly present as viral genomic dsRNA and less common as viral ssRNA, i.e., viral mRNA [121]. This indicates that after the peak phase of infection are the transcription and translation of viral genes low, but this is not reflected when performing PCR analysis of viral RNA that normally will not distinguish between ds and ssRNA. The histopathological changes in relation to HSMI are detected after the acute phase. The viral kinetics of PRV-1 infection indicate that quantitative analysis of PRV-1 RNA after peak infection should be interpreted with caution and if possible, accompanied by protein analysis. In the material used in Papers I and III, samples were obtained at slaughter and probably well beyond the acute infection phase. However, abundant PRV-1 signal was detected by IHC in the changes. A panel of PRV-1 antibodies targeting capsid proteins

σ1 and µ1C and the non-structural protein µNS showed persistent and replicating virus within the changes. This can explain the chronic nature of focal melanised changes, but in comparison with the viral kinetics of PRV-1 infection and subsequent HSMI, these results are unexpected. These results indicate that PRV-1 infection can be related to several different conditions and a spectre of different cells. In the case of melanised focal changes, the macrophages (both M1 and M2) and melano-macrophages are essential, while these cells have a minor role in HSMI, where erythrocytes and T cells are important [21, 85].

10.2.3 Real-time quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR is an important method in biomedical and fundamental research. It is a refinement of the original PCR method where it is possible to determine the presence and amount of target RNA in a sample, keeping the sensitivity of the method. In RT-qPCR, the RNA is reverse transcribed to cDNA before qPCR. The amplified DNA product is quantified after each round of amplification, allowing determination of the transcription level. This is achieved by using a specific fluorescent reporter, whose signal is analysed and further calculated as relative expression by using a housekeeping gene as reference. The method is routinely used in fish research and diagnostic work and is thus well-known and established as a reliable method. Some of the RT-qPCR (targeting pathogens) was conducted by Patogen Analyse in Ålesund, an approved and certified fish health laboratory.

In quantitative gene expression analysis, two different methods are commonly preferred: SYBR Green and TaqMan, both of which were used in the studies included in this thesis. SYBR Green is based on binding of fluorescent dye to dsDNA whereas the TaqMan method is based on fluorescent labelled oligonucleotide and the exonuclease activity of the Taq polymerase enzyme. This technical difference has several advantages but contributes to why TaqMan probes are more expensive. Also, if the PCR is poorly designed, SYBR Green will detect all dsDNA, which can lead to erroneous results.

In the studies of this thesis, RT-qPCR was used to analyse the expression of viral-, antiviral- and immune genes (Paper I-IV). The primers used have been evaluated and used in numerous studies and are also used in the daily fish health monitoring of infectious diseases. Some of the

analyses for PRV-1 expression were done in-house (Paper I and III), in addition to the analysis for immune- and antiviral genes in Paper III.

10.3 RESULTS AND GENERAL DISCUSSION

In the beginning of this study, the possible link between red and melanised focal changes had not been established, although industry representatives assumed this to be the case. In fact, "red spots" or red focal changes had never been reported in any peer-reviewed publication. There was no information on their macroscopic or histologic appearance and their prevalence was unknown. Melanised focal changes had previously been characterized as a polyphasic necrotizing myopathy; however, the histological heterogeneity of such changes had not been classified with regards to occurrence and severity. Also, the role of any pathological agent in the development of focal melanised changes was unknown. Thus, most of the work conducted in the present thesis was aimed at detecting, if any, pathological agent(s) and further investigating their/its role in the pathogenesis, and, to characterize the putative histological heterogeneity of the changes. The strong association between the changes and PRV-1 presence (shown by IHC in Paper 1) required further investigation with respect to the role of the presence of PRV-1 in the condition. By combining the work on PRV-1 with investigating the immune response in red and melanised focal changes and a thorough study of the role of the macrophages, this work contributes to revealing important aspects of the pathogenesis of melanised focal changes in Atlantic salmon.

10.3.1 Histological investigations of red, transient, and melanised focal changes

Initial histological investigations of red focal changes collected at slaughter (Paper I) revealed an acute haemorrhagic necrotic myositis. In contrast to melanised focal changes, there were no organised granulomas and melano-macrophages were generally not present. In addition, transient stages were also identified at slaughter, appearing macroscopically as both red and melanised. As previously stated, neither red focal changes, nor transient stage changes had ever been reported following investigation by histological methods. The transient stage changes were best described as containing features of both the acute and the chronic manifestation, i.e., with haemorrhage, fibrosis, often granulomatous inflammation and melano-macrophages. Based on

the histological characteristics of the different manifestations, a timeline was proposed for the condition, initiated as an acute focal haemorrhagic myositis, progressing into a transient stage, and ultimately developing into a chronic melanised change with granulomatous inflammation. The only consistent finding in all changes was the presence of PRV-1 and thus PRV-1 was hypothesised to be the driving force of the condition. However, the results did not show that this was the order of events. Only a controlled experiment inducing changes in a similar order and initiated by PRV-1 infection would prove this development. Such an experiment was attempted in Paper II but failed to induce any detectable changes (discussed further in 10.3.2).

The histological investigations were widely expanded in Paper II, from only including changes collected at slaughter in Paper I, to samples collected at different timepoints throughout the entire seawater phase in Paper II. This expanded sampling protocol revealed a hitherto unknown histological heterogeneity in melanised focal changes and thus a classification system was developed. This resulted in nine different categories: (a) no histological changes; (b) melano-macrophages in the endomysium between apparently intact myocytes; (c) fibrosis in the endomysium without detection of melano-macrophages; (d) fibrosis in the endomysium with melano-macrophages; (e) melano-macrophages, fibrosis and presence of inflammatory cells in the endomysium; (f) (anticipated) old scar tissue with presence of inflammatory cells; (g) old scar tissue with presence of inflammatory cells and melano-macrophages; (h) focal granulomatous inflammation with presence of melano-macrophages; and (i) diffuse granulomatous inflammation with myocyte necrosis and myocyte regeneration and presence of melano-macrophages.

As the fish in this trial were PRV-1 negative at the time of sea transfer, the onset of PRV-1 infection was addressed in relation to the development of different changes. Indeed, the most severe histological categories corresponded well with increasing macroscopic severity grade. Also, the most pronounced changes with diffuse granulomatous inflammation were only seen in PRV-1 infected fish. However, the study showed that red focal changes and even low grade melanised focal changes could occur prior to PRV-1 infection, challenging the pathogenesis proposed in Paper I (discussed further in 10.3.2). Also, changes could appear macroscopically identical, but be very different histologically. Indeed, some changes appeared with normal tissue

architecture or only with some limited fibrosis but were still registered macroscopically as melanised focal changes. This finding highlights the need for histological analysis when investigating this condition. The different histological manifestations might just represent different stages of the changes, all originating from the same cause(s), or they could be the result of different triggers leading to different histological characteristics.

10.3.2 Detection of PRV-1 in red and melanised focal changes

From early on, vaccination was thought to be a driver of melanised focal changes [5]. This assumption was strengthened with the detection of adjuvant oils in such changes [122]. Therefore, intramuscular deposition of oil adjuvant was targeted as the main cause of melanised focal changes. However, reports on melanised focal changes in unvaccinated farmed salmon dismissed vaccination as the only probable cause [113, 114]. Besides vaccination, the possible involvement of infectious agents such as parasites, fungi, bacteria or viruses has been subject for investigation. The characteristic histopathological changes could very well fit with infectious agents such as bacteria or fungi, a probable cause of granulomas. However, all previous studies have failed to show a consistent finding of microorganisms or viruses within the changes. PRV-1 was especially interesting in this regard, being a virus known to infect both heart and skeletal musculature causing necrosis and inflammation, commonly recognized as HSMI. HSMI was first associated with PRV-1 and it was later shown a causal relationship between the disease and the virus. The work by Finstad/Wessel and co-workers was especially important for this study, producing several antibodies targeting PRV-1, which laid grounds for the initial detection of PRV-1 in melanised focal changes. With antibodies available, testing of histological sections using IHC became possible, which had never been attempted before. Although Larsen et al. [108] did investigate the possible presence of PRV-1 in focal melanised changes using RT-qPCR, their results were negative. However, antibodies or ISH protocols for PRV-1 were not available at that time and they could not verify this result in any other ways. In addition, the primer design and PCR conditions are not completely described in materials & methods, making this study difficult to compare with other RT-qPCR studies on PRV-1. Also, the results conflict with all other data in later studies and should thus be interpreted with caution.

In the first paper of this study, PRV-1 was detected in all red, transient and melanised focal changes investigated. These changes were obtained at slaughter. Due to the consistency in the results, an association between PRV-1 infection and focal muscle changes was concluded. PRV-1 was also suggested to be a premise for development of melanised focal changes. However, it was also stressed that the results did not establish that PRV-1 could initiate the process leading to the formation of red focal changes. This was further investigated in Paper II. PRV-1 negative fish were followed after sea transfer with regards to the onset of PRV-1 infection and the development of red, transient and melanised focal changes. Interestingly, red focal changes and low-grade melanised changes were shown to occur prior to PRV-1 infection, arguing that PRV-1 is not a premise for these changes. However, PRV-1 was always present in high grade melanised changes with granulomatous inflammation. This finding leads to the question of the significance of PRV-1 in the pathogenesis. As melanisation is a general immune mechanism, several different triggers can initiate this process, and it thus seems just to indicate that different underlying causes can give rise to melanised focal change, PRV-1 infection being one of them. The detection of PRV-1 with two different antibodies (targeting structural capsid proteins) and with genetic methods (RT-qPCR and ISH) has undoubtedly shown the presence of PRV-1 within different muscle changes. IHC demonstrated PRV-1 positive macrophage-like cells, but also melano-macrophages were occasionally immuno-positive. Detection of the non-structural protein μNS in melanised (and even some red) focal changes showed replication of virus within the changes, which is strong evidence for PRV-1 as a persistent antigen and a constant trigger of inflammation, ultimately leading to the formation of granulomas. Replicating virus was even detected within well-organized granulomas in macrophage-like cells and in multinucleated giant cells, whilst no other infectious agents were found. Based on these results, a possible pathogenesis is that red focal changes in virus infected fish are suffering from PRV-1 infected extravascular erythrocytes which are degraded *in situ*. The degradation of infected extravasated red blood cells causes a further local inflammatory reaction towards the virus. Macrophage-like cells and melano-macrophages are recruited, but instead of clearing the infection, the virus persists and replicates within the changes, ultimately leading to the formation of granulomas. This possible line of

events is also supported by the results on macrophage polarization in Paper IV (further discussed in 10.3.3).

Although the results on PRV-1 presence and replication are strong and argue for PRV-1 as a key feature of melanised focal changes, the results only show correlation and not causation. An experimental infection trial was designed aiming at inducing red and melanised focal changes by injecting PRV-1. PRV-1 was injected (in several ways) in a trial lasting for 18 weeks. However, this experiment failed to induce any detectable changes. The results from the field trial in Paper II clearly demonstrated that PRV-1 infection is not sufficient to trigger the onset of the condition and probably not the cause for the initial red focal changes. Some "factor X", possibly production-related, in the farms may be necessary to initiate the condition, and without this factor X, it was not possible to induce changes experimentally in research facilities. Red focal changes also occur in non-infected fish and the cause of such changes remains unknow. It is possible that such changes can heal completely or become low-grade melanised changes. To study the outcome of different types of changes, a model of induction is necessary.

10.3.3 The immune response in red and melanised focal changes

Studying the immune response in red and melanised focal changes was necessary to understand further their pathogenesis. Red focal changes had never been investigated in this respect. Most red changes had some slight histological variation in the content of hemorrhage, necrosis, inflammatory cells and/or fibrosis, but compared with melanised changes, the red focal changes were rather homogenous. RT-qPCR, IHC and ISH results revealed that the red changes were dominated by innate immune responses, consistent with acute changes. The anti-inflammatory cytokine IL10 was significantly downregulated in the red changes and identified as a key regulator of the immune response. The absence of IL10 expression was in line with increasing trends in several pro-inflammatory cytokines (TNFα, IFNγ and IL1). Melanised focal changes, on the other hand, showed features consistent with activation of adaptive and prolonged immune responses. Here, a modest increase in IL10 was detected. A characteristic feature of high-grade melanised changes was the presence of granulomas. Not only bacterial (especially mycobacteria), fungal and parasitic infections can cause granulomas; some viruses also have this ability. Viruses

reported to be associated with the development of granulomas in humans include cytomegalovirus, Epstein-Barr virus and measles morbillivirus [123]. In veterinary medicine, feline coronavirus, causative for feline infectious peritonitis (FIP), a systemic disease in cats, is characterised by granulomas and granulomatous vasculitis [124]. Granulomas can be regarded as mere end-stages of the immunological response; a default when all other attempts to eradicate the persistent agent fails. However, granulomas have a significant protective function by isolating foreign agents in a focal, chronic inflammatory tissue response composed mostly of macrophages [123]. This development is evoked by persistent, poorly degradable substances and is especially useful in the case of replicating intracellular invaders such as PRV-1. Indeed, the results in this study have shown the consistent presence of PRV-1 in high grade melanised changes and even within highly organised granulomas. The presence of the non-structural viral protein µNS demonstrated replication of PRV-1 *in situ* in the changes. This is a very important result, arguing for the role of PRV-1 as the chronic trigger of inflammation.

When comparing chronic inflammatory reactions of salmonids, melanised focal changes and vaccine-induced peritonitis are rather similar in their appearance. It seems that melano-macrophages are common in certain inflammatory reactions, especially those leading to the formation of granulomas. A trait to such conditions in fish is the presence of a persisting agent, which can be a range of different agents, including foreign objects [125], vaccine-adjuvant and bacterial or possibly viral antigens, among others [5, 34]. In contrast with these inflammatory conditions, chronic enteritis, such as SBMIE, is different in histological appearance [126]. Indeed, SBMIE is probably an initial hypersensitivity reaction towards saponins which over time can progress to a granulomatous inflammation [127], however, organised granulomas are not evident. Neither is the presence of melano-macrophages.

As stated above, macrophages are the key cells of a granulomatous inflammation and they are also the main cell in the formation of granulomas. Thus, the role of the macrophages in the development of melanised focal changes was investigated in-depth in paper IV. The macrophage is a rather pleomorphic cell type, which can adopt a distinct phenotype in response to a changing microenvironment. For instance, in granuloma they can fuse into multi-nucleated giant cells, or they can develop into epithelioid cells. Therefore, macrophages can be involved both in acute

destruction of invading pathogens and in tissue homeostasis and repair. MHC class II positive cells were detected in moderate to high amounts in all changes investigated in Paper III, and most of these were presumed to be macrophage-like cells based on their appearance and distribution. In Paper IV, the macrophages were further analysed in depth by FISH targeting genes specific for different macrophage phenotypes (M1 – iNOS2 and M2 – Arg2. See 7.4.3 for more information). Red and melanised focal changes from both PRV-1 infected and non-infected fish were investigated. The results showed a clear polarisation of the macrophages in PRV-1 infected fish, with mainly pro-inflammatory M1 macrophages in red changes and anti-inflammatory M2 macrophages (cells involved in tissue repair) in melanised changes. As similar trends were not detected in non-infected fish, macrophage polarisation was linked to the presence of PRV-1, but also to the formation of melanised focal changes. This underlines the important role of the macrophages in the condition, and possibly also why PRV-1 infected fish are prone to develop more severe changes with granulomatous inflammation.

10.3.4 Implications for the aquaculture industry

Prior to this study, all melanised focal changes were regarded as identical, only with slight variation in size and pigment intensity. “Red spots” were merely regarded as bleedings occurring in the same region as melanised focal changes, but it was unknown whether the changes were related. Pigmentation of the filet in general was regarded as a quality problem and no infectious agents were associated with the condition.

The results of this study showed the plausible progression from acute red to chronic melanised focal changes, which is valuable information for understanding the pathogenesis of this condition. Further, PRV-1 infection was identified as a risk factor for more severe changes (high-grade melanised changes with granulomas). Due to the widespread presence of PRV-1 and the usual low morality (and economic loss) from HSMI, fish health personnel have perhaps not regarded PRV-1 infection as a considerable health problem. With the results from this study at hand, melanised focal changes can also be regarded as health problem and even as a disease, and prophylactic health work on PRV-1 could potentially protect farmed fish from both HSMI and severe, melanised focal changes.

However, according to health surveys and viral screenings, the present situation is that all farmed fish in Norway are infected with PRV-1, either during the freshwater stage or at sea. There are no reports of fish uninfected with PRV-1 making it all the way to slaughter. However, immunization of fish, especially targeting viral diseases, is continuously improving and vaccination against HSMI is not impossible in the future. A vaccine would possibly reduce the problem of melanised focal changes.

In addition, the field study in Paper II provided the industry with prevalence data on both red and melanised focal changes at different time-points throughout the seawater period. These data are extremely valuable as the data show the progression of the condition, which was unknown prior to this study.

11 CONCLUSIONS

This thesis has provided novel information on pathogenesis of focal melanised changes in farmed Atlantic salmon and increased our understanding of the development of the condition. The papers (Paper I - IV) addressed the hypotheses and sub goals defined in Aims of the study, as summarised below.

The development of this project hinged on an initial hypothesis, namely that PRV-1 is present in melanised focal changes in the filet at slaughter. The verification or falsification of this hypothesis would determine the subsequent course of events. To reveal the potential presence of the virus, investigations of the changes were conducted with the at that time available methods, i.e., IHC and RT-qPCR. Melanised focal changes from different fish populations collected at the abattoirs were examined for the presence of PRV-1 (Paper I). The primary hypothesis was verified as PRV-1 was shown to be a consistent finding in all changes investigated (Paper I). PRV-1 was detected in macrophage-like cells, erythrocytes, and melano-macrophages, both diffusely in the inflamed tissue and within highly organised granuloma. In addition, the changes were screened for other potential pathogens, such as salmonid alphavirus and infectious pancreatic necrosis virus, but these investigations were negative. Special stainings (and ISH) for bacteria were also negative in the changes.

Following the initial detection of PRV-1 in melanised focal changes (Paper I), the subsequent goal thus became to investigate the potential role of PRV-1 infection in the development of the condition. As industry representatives had reported a modest occurrence of "red spots" (red focal changes) in the same anatomical location of the filet as melanised focal changes, and these red focal changes were presumed to be an initial manifestation of the condition, a second hypothesis was postulated: PRV-1 infection causes acute red focal changes in the musculature, which can progress into a chronic inflammatory condition with the local presence and accumulation of melano-macrophages, thus developing into melanised focal changes. Seven research goals were defined to test this hypothesis, each of which is answered below.

1. To determine the presence of PRV-1 in red focal changes using both protein and gene expression analysis (Paper I and II):

   Red focal changes collected at the abattoirs were screened for relevant viruses by RT-qPCR and IHC and subjected to special histological stains for detection of bacteria (Paper I). The only consistent finding was the presence of PRV-1. PRV-1 was mainly detected in macrophage-like cells but also in erythrocytes.

   In sequential samplings of fish throughout the seawater period, red focal changes were sampled from PRV-1 negative fish in the first months after sea transfer (Paper II). Thus, it appeared that red focal changes could occur both with and without PRV-1 infection.

2. To characterise red focal changes macroscopically and microscopically (Paper I and II):

   Red focal changes collected at the abattoirs were determined to occur in the cranio-ventral part of the filet, i.e., the same location in which melanised focal changes are most prevalent (Paper I). Foci extended from 1–3 cm and could involve 2–6 myotomes. Incisions through foci revealed extension of the haemorrhage deep into the muscle. The changes were histologically characterised as an acute haemorrhagic necrotic myositis. The degree of inflammation varied between the changes. Melano-macrophages were generally not present and organised granuloma were never

detected. Fibrosis was present to some degree, mainly associated with vacuoles of unknown role and function.

In sequential samplings of fish throughout the seawater period, red focal changes occurred with a low prevalence in all samplings, both prior to and after PRV-1 infection (Paper II). Changes of all macroscopic grades (1-3) were present in all samplings and the histological findings showed some minor variation that seemed unrelated to the macroscopic appearance. The general histological description with haemorrhage, necrosis and a varying degree of inflammation was valid for all red focal changes investigated.

3. To reveal key features of red and melanised focal changes and investigate whether the changes are different (time-related) manifestations of the same condition or not (Paper I and II):

   Red and melanised focal changes collected at the abattoirs were determined to represent likely acute and chronic stages, respectively, of the same condition. This was based on macroscopic observations of transient stage changes appearing both red and melanised. Histologically, these changes were characterised by haemorrhage and muscle necrosis, but also a differing presence of melano-macrophages (Paper I and II). This strengthened the link between the two types of muscle changes, indicating a development from one form to the other.

4. To investigate the frequency and development of red and melanised focal changes in relation to the onset of PRV-1 infection in the seawater phase of farmed salmon (Paper II):

   The frequency of red focal changes was shown to be consistent throughout the seawater phase at about 4%, while melanised focal changes showed an increasing trend, starting at 6% and ending at about 30% at slaughter. Both red and melanised focal changes occurred in all samplings (with and without PRV-1 infection), from early after sea transfer until the final sampling at slaughter. However, in contrast to red

focal changes, melanised focal changes were of lower severity in the first samplings (PRV-1 -) and more severe in later samplings (PRV-1 +). This coincided with the histological classification of melanised focal changes (category 1-9), where most category 9 changes (diffuse granulomatous inflammation) were seen in grade 3 changes, all with PRV-1 presence. Additionally, all these changes were observed in the last three samplings prior to slaughter, showing a progression in severity over time. The onset of PRV-1 infection also fitted well with the development of more severe changes, as the population gradually became infected from 23 weeks post sea transfer until week 48 when all fish were infected.

5. <u>To induce experimentally red and melanised focal changes in Atlantic salmon in a challenge study with PRV-1 and study their development (Paper II):</u> Groups of fish held at Veso Vikan research facility were injected with PRV-1 in several ways, including i.p. injection with pelleted blood containing high loads of PRV-1, i.p. injection with PRV-1 infected erythrocytes, and both i.m. and i.p. injection with purified PRV-1 (heat treated). Control groups were injected with either PBS or *Renibacterium salmoninarum* and one additional group was left untreated. The experiment ran for 18 weeks post-challenge with sampling of six fish every third week.

   The experiment failed to achieve the research goal, as no red or melanised changes occurred in any of the groups. Thus, PRV-1 infection alone seems insufficient to induce the condition.

6. <u>To reveal possible inflammatory responses in red focal changes from slaughter size fish (PRV-1 positive fish), and if present, characterize and compare them with the inflammatory responses in melanised focal changes (Paper I and III):</u> The degree and character of the inflammatory reactions in red and melanised focal changes were assessed histologically (HE staining) in Paper I, as described above (sub-goal 2, 3 and 4). The immune cells in red focal changes collected at slaughter (PRV-1 infected fish) were further characterised by more refined methods (IHC, ISH and RT-qPCR) in Paper III, showing a somewhat varied immune response, but dominated by

MHC class I/II positive cells and also focal infiltrates of T cells (CD3 and occasionally CD8 positive cells). Few to moderate amounts of B cells were detected. Trends in the RT-qPCR showed upregulation of genes related to innate immunity in the red focal changes, whereas genes related to adaptive immunity were upregulated in the melanised focal changes. An important result was the significant downregulation of the anti-inflammatory cytokine IL10 in all red changes, shown as a key immunological trait for the condition.

7. To investigate the potential role of macrophage polarity and the cell mediated immune response with regard to the development of red and melanised focal changes in both PRV-1 infected and non-infected fish (Paper IV): In the further characterisation of the inflammatory microenvironment, the macrophage heterogeneity and the cell mediated immune response were investigated in red and melanised focal changes, from both PRV-1 infected and non-infected fish (same material as in Paper II). In PRV-1 infected fish, the abundant presence of iNOS2-expressing M1-polarized macrophages in red focal changes demonstrated a pro-inflammatory microenvironment (consistent with IL10 downregulation, Paper III). These iNOS2 expressing cells showed a high level of co-localisation with PRV-1 presence. On the other hand, in melanised focal changes (also from PRV-1 infected fish) few iNOS2-expressing cells were detected, but instead a relatively high number of arginase-2-expressing anti-inflammatory M2-polarised melano-macrophages were present. This indicated an ongoing healing process. In both red and melanised changes, co-localization of PRV-1 and cells expressing CD8+ and MHC-I suggested a targeted immune response in the changes, probably towards PRV-1 infected cells. In non-infected fish, there was no trend showing macrophage polarity, nor a significant induction of cell mediated immunity. Importantly, the melanised focal changes in the non-infected fish were also of lower grade (without granulomas), as high-grade melanised changes never occurred prior to PRV-1 infection (as classified in Paper II).

Based on these results, macrophage polarisation and a targeted cell mediated immune response seem to correlate with PRV-1 infection when present, resulting in the development of red and high grade melanised focal changes with granulomatous inflammation.

The hypothesis, stating that PRV-1 infection causes acute red focal changes in the musculature, which can progress into a chronic inflammatory condition with the local presence and accumulation of melano-macrophages, thus developing into melanised focal changes, is rejected based on the results from Paper II. The study revealed that red and mild forms of melanised focal changes could occur prior to viral infection. This is not in accordance with the hypothesis. However, the study revealed that the impact of PRV-1 infection seemed to affect the severity of melanised focal changes, where persistence of viral antigen within the changes was seen in association with unresolved granulomatous inflammation with melanisation. Replication of virus *in situ* was also shown by IHC, showing abundant presence of the non-structural viral protein µNS (Paper III). These events must be seen in context with the heterogeneity of the macrophages (Paper IV), as PRV-1 infection appears to be the driving force in polarization of the macrophages. A targeted cell mediated immune response in PRV-1 infected changes also supports this assumption (Paper IV).

12 FUTURE PERSPECTIVES

This thesis focused on the influence of PRV-1 infection on the development of red and melanised focal changes. Although novel features of the pathogenesis have been revealed, key questions related to this condition remain unanswered. The main challenge seems to be to determine the initial cause of red focal changes. This thesis has established that red focal changes are the initial manifestation of the problem. The histological classification as outlined in Paper II showed that melanised focal changes can macroscopically appear quite similar with the exception of the most severe forms, but still be histologically very different. With respect to the red focal changes, they represent acute changes/forms which generally appear quite similar. This indicates a common cause for these changes. "Focal" is a key word in this respect. The focal delimitation of the change argues for some local factor of which many possible explanations have been suggested. As discussed in Paper II, the cranio-ventral part of the filet is the fattiest area, with values twice as high as more caudal parts. The huge amount of fat in the fish diet could lead to health problems and one could speculate that fat transport in blood (as lymphatic vessels are not recognised) could cause problems in this particular region and even occlude vessels and cause haemorrhages. This has been observed in the gill capillaries (personal communication, prof. em. Agnar Kvellestad).

Another suggested explanation is the close proximity between red/melanised focal changes in the filet and the stomach. The changes are frequently seen directly underneath the peritoneal wall and continue into the depth of the filet. Almost continuous packing of the stomach (with pelleted food) during the seawater phase would cause the stomach to distend and potentially lead to continuous pressure on the inner abdominal wall, ultimately leading to a trauma/haematoma or ischaemia. Yet another possible factor is the proximity between the pectoral fins and the muscle changes. The fins are frequently worn and in farmed fish, and wear of the fins could potentially cause local trauma in the area, including the surrounding musculature. Damaged fins would also be a gateway for infectious agents. Future work should aim to reveal the mechanisms involved in the development of red focal changes, as the key to reduce melanised focal changes most likely lies in reducing this initial manifestation.

A common feature for most of the red focal changes, was the presence of extravascular erythrocytes, i.e., haemorrhage. The erythrocytes of fish are special in the sense that they are nucleated and thus contain the transcriptional and translational machinery enabling virus replication. Additionally, they can form rosettes to facilitate the clearance of pathogens by macrophages [56] and also produce cytokines or specific signalling molecules in response to binding, and thus, actively take part in immune responses [128]. The immunological role of erythrocytes in red focal changes was not addressed in this thesis but should be investigated in future studies, as their role as cells with immune functions might be important for the condition.

An important unknown to address in future research is the activation of the pigmentary system, its likely interaction in innate and adaptive immunity, and especially how the melano-macrophages are activated. First, it would be important to establish some basal information on melano-macrophages, e.g., their development in relation to other leukocyte populations. Are melano-macrophages a sub-type of macrophages capable of melanin-production or do all macrophages in fish have this ability under the right stimulation? If we study melano-macrophages and other immune cells in context, we will understand much more about their potential functions and impact. By ISH, gene transcripts of the melanin synthesising pathway (Tyrosinase, MITF, TyRP) can be targeted to determine the onset of melanogenesis and see when and how the cells are activated. Preliminary data indicate that amelanotic macrophage-like cells can express these genes, and under the right conditions, possibly start producing melanin. A solid establishment of the possible functional interactions between the pigmentary and immune systems would be a major achievement. Another vital point in this work would be the interaction with virus. As seen in Papers I and IV, melano-macrophages were PRV-1 positive, but it is not known how the virus is further processed within the cell. Future ultrastructural investigations could answer these highly important questions.

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14 ENCLOSED PAPERS I-IV

I

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VETERINARY RESEARCH

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Piscine orthoreovirus (PRV) in red and melanised foci in white muscle of Atlantic salmon (*Salmo salar*)

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Abstract

Melanised focal changes (black spots) are common findings in the white skeletal muscle of seawater-farmed Atlantic salmon (*Salmo salar*). Fillets with melanised focal changes are considered as lower quality and cause large economic losses. It has been suggested that red focal changes (red spots) precede the melanised focal changes. In the present work, we examined different populations of captive and wild salmon for the occurrence of both types of changes, which were investigated for the presence of different viruses by immunohistochemistry and RT-qPCR. The occurrence of red or melanised foci varied significantly between the populations, from none in wild fish control group, low prevalence of small foci in fish kept in in-house tanks, to high prevalence of large foci in farm-raised salmon. Large amounts of *Piscine orthoreovirus* (PRV) antigen were detected in all foci. No other viruses were detected. Red focal changes contained significantly higher levels of PRV RNA than apparently non-affected areas in white muscle of the same individuals. Some changes displayed a transient form between a red and melanised pathotype, indicating a progression from an acute to a chronic manifestation. We conclude that PRV is associated with the focal pathological changes in the white muscle of farmed Atlantic salmon and is a premise for the development of focal melanised changes.

Introduction

Farmed Atlantic salmon may develop melanised focal changes in the white muscle [1]. The condition represents a considerable challenge for the industry with as much as 20% of the fillets at Norwegian processing plants reported to be affected [2]. Changes typically appear as distinct areas within the white muscle, frequently located in the cranio-ventral and craniodorsal regions of the abdominal wall, but may also be found elsewhere in the musculature [1,3]. This condition causes large economic losses as fillets with pigmentation disorders are downgraded. Melanised focal changes are common in farmed Atlantic salmon all along the Norwegian coast. However, the prevalence is negligible in farmed rainbow trout (*Oncorhynchus mykiss*) although they are produced under similar conditions (H. R. Sæbø, Lerøy, personal observation). Additionally, there are no reports of melanised focal changes in wild-caught salmon (E. Sterud, Norwegian Salmon Rivers, personal communication). Melanised muscle changes were reported in a study addressing the tagging of wild salmon [4], but with a visible physical foreign body present, this condition is not comparable with melanised focal changes as described previously [1].

The discolouration is attributed to melanin-producing leukocytes referred to as melano-macrophages [5-7]. Normally, such cells are prevalent in lymphoid organs of fishes [6,7], but accumulation of melano-macrophages may be part of chronic inflammatory responses such as peritonitis induced by oil-adjuvanted vaccines [8]. Macrophages use oxidation to inactivate pathogens, and there is a particular need for protection against oxidative damage in chronic inflammatory processes. In contrast to mammals, populations of leukocytes in ectothermic vertebrates produce melanin, which is a powerful anti-oxidant. It has been proposed that these melanin-synthesising mesenchymal-derived cells could be classified as melanocytes [9].

The aetiology of the focal melanisation in the white muscle has remained obscure. It was previously suggested

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that the changes might occur due to chronic inflammatory processes induced by dislocation of oil-adjuvanted vaccine components [1]. Nevertheless, later studies showed that also un-vaccinated fish may develop similar lesions at the same prevalence, and the hypothesis of a vaccine-induced aetiology was abandoned [10-12]. Other explanations that have been suggested point towards mechanical trauma due to handling of the fish, causing focal intramuscular haemorrhages that later melanise. However, these assumptions have not been documented by histological investigations. Besides, intramuscular haemorrhages and haematoma do not initiate granulomatous inflammation.

Melanised, but not red, focal changes have been investigated by different morphological and transcriptional approaches in a number of studies. Histological examination has revealed granulomatous inflammation in myotomes and myosepta, frequently containing large vacuoles surrounded by melano-macrophages. T-cells and MHC class II^+ cells dominate the leukocyte infiltrates in the affected tissues. Nevertheless, myocytes seem to be the primary affected cell type, and therefore the term "chronic polyphasic necrotizing myopathy" has been applied [1,3]. Melano-macrophages are either found in clusters or scattered throughout the changes with melanin produced in situ, making this granulomatous reaction a unique inflammatory response [3]. The characteristic histological appearance indicates that the pigmentation caused by chronic inflammatory response is due to the underlying necrotizing myopathy. The granulomatous inflammation indicates a chronic antigenic stimulation, for instance a foreign-body reaction as suggested before [1] or a chronic infection. However, the aetiology of this myopathy has remained enigmatic. Several viral infections in farmed salmonids are chronic. Both *Piscine orthoreovirus* (PRV) and Salmonid alphavirus (SAV) cause skeletal muscle inflammation in Atlantic salmon [13,14]. PRV-caused changes are most prominent in red muscle, while SAV-caused changes are found in both red and white muscle [13,15]. SAV is widespread in southern parts of the Norwegian Coast and only sporadically found in the northern part. In contrast, melanised focal changes are found in farmed salmon including areas that never have experienced SAV infections.

Heart- and skeletal muscle inflammation (HSMI) is associated with PRV, and is characterized by severe pancarditis, inflammation and necrosis of red myocytes in Atlantic salmon. Pathological changes have also been described in white myocytes [16]. Several PRV-screenings based on reverse transcription qPCR (RT-qPCR) have shown that PRV is almost ubiquitously present in seawater-farmed Atlantic salmon in Norway. PRV has been detected in apparently healthy fish throughout the production cycle [17], and has also been found in wild salmon [18,19]. Erythrocytes have been found to be the central target cells for PRV, implying that the virus can be found in any organ in an infected individual [20]. Infected erythrocytes contain globular inclusions, i.e. viral factories that contain PRV protein and dsRNA [21,22]. The gene expression pattern due to PRV infection in salmon erythrocytes includes large-scale changes of immune regulators [23].

In this study, we examined samples from groups of farmed, wild and in-house Atlantic salmon for the occurrence of red or melanised focal white muscle changes and tested for the presence of different viruses. Here we show large amounts of PRV in such changes.

Materials and methods

Groups

The study included eight different fish populations (A-H). Groups A-D were sea-farmed fish collected at different farm sites in west and north on the Norwegian coast. Groups A and B were selected because of high and low prevalence of melanised focal changes, respectively. As red focal changes are much less frequent compared with melanised focal changes, an additional sampling was conducted where only muscle samples were collected on RNA*later* and formalin (Group C). Group D consisted of archived paraffin-embedded material of melanised focal changes sampled in 2008. Groups E-G were fish collected at Institute for Marine Research (Matre Research Station). Group E consisted of vaccinated fish kept in sea cages. Group F was vaccinated fish kept in sea water in-house tanks together with a group of unvaccinated fish (Group G). The sea-water was obtained from 70 meters depth. Group H was wild Atlantic salmon caught with dip-net at Hellefossen in the Drammen River following their return for spawning, approximately 400 km away from the nearest commercial sea-farm. Details for the different groups are presented in Table 1.

Sampling

First, peripheral blood was collected in vacutainer tubes containing heparin. The fish were subsequently bled to

Table 1 Group information

Group	Fish (n)	Age (y)	Broodstock	Water	Length (cm)	Weight (g)
A	25	2	Farmed	Sea	n/a	4144
B	35	2	Farmed	Sea	n/a	4056
C	10	2	Farmed	Sea	n/a	4696
D	26	n/a	Farmed	Sea	n/a	n/a
E	20	3	Farmed	Sea	73.5 ± 5.6	4908 ± 1009
F	42	3	Farmed	Sea	60.5 ± 5.5	2763 ± 815
G	42	3	Farmed	Sea	57.4 ± 7.7	2953 ± 747
H	10	n/a	Wild	Fresh*	81 ± 12.5	5020 ± 2210

*returning to river.
(n/a - Not available).
Locations: **A** – Northern Coast. **B, C**, **D**, **E, F** and **G** – Western Coast. **H** – Drammen River, South-east.

death and opened by an abdominal incision. The organs were examined and the fish were filleted and the muscle examined for changes. Fish with and without visible muscle changes were used for the subsequent analysis. Samples from white muscle and spleen were collected on RNA*later* and 10% phosphate-buffered formalin. From individuals where muscle discolouration were detected, either as red or melanised focal changes, samples from affected areas and from macroscopically non-affected muscle were collected as described above. Hearts from all fish in Groups A-B and E-H were collected in formalin. All fish included in this study were anaesthetised prior to sampling, in line with regulations of the Norwegian Directorate of Fisheries.

Anamnestic information

Groups A-F had been intraperitoneally immunised, while the fish in Group G was left un-vaccinated. No anamnestic information was available for the wild fish (Group H). Groups A-E had been routinely deloused, whilst Groups F-G (kept in in-house tanks) received no such treatments. Group A was diagnosed with severe outbreak of HSMI with 9% mortality 5 months prior to sampling. HSMI had been suspected in Group C, but was not investigated further. Despite being vaccine-naïve, no diseases were recorded in Group G, and no mortality was observed during the observation period.

Histology and immunohistochemistry

The tissue samples were fixed in formalin for 24–48 h, and next dehydrated and embedded in paraffin according to standard procedures. The slides were cut 2 μm thick and mounted on glass slides (Superfrost®, Mentzel, Braunschweig, Germany), incubated for 24 h at 37 °C, de-waxed in xylene and rehydrated through graded alcohol baths. Sections were stained according to standard procedures with haematoxylin and eosin (HE), with van Gieson's method for detection of collagen and with Perl's Berlin blue for detection of ferric iron. The slides were mounted with polyvinyl alcohol media (Ullevål Apotek, Oslo, Norway).

The paraffin-embedded material was also used for immunohistochemical investigations. The different steps were performed at room temperature unless otherwise stated. The sections were cut 4 μm thick and mounted on glass slides (Superfrost©; Mentzel, Braunshweig, Germany), incubated for 24 h at 37 °C and thereafter for 30 min at 58 °C, de-waxed in xylene and rehydrated in graded alcohol baths before transferring to distilled water. Sections were next autoclaved in 0.01 M citrate buffer, pH 6.0 at 120 °C for 10 min to retrieve antigens, followed by treatment with phenylhydrazine (0.05%; Sigma-Aldrich, St. Louis, MO, USA) for 40 min at 37 °C to inhibit endogenous peroxidase. The slides were rinsed three times in phosphate-buffered saline (PBS).

Nonspecific binding was prevented by adding PBS with 2% (v/v) normal goat serum plus 5% (w/v) bovine serum albumin (BSA) or 5% (w/v) skimmed dry milk. The primary antibody –in tris-buffered saline (TBS) with 1% BSA - was added and incubated for 30 min. The sections were then rinsed three times in TBS and, and further incubated with a secondary antibody (EnVision© System kit; Dako, Glosrup, Denmark) for 30 min. The slides were again washed three times in TBS, and the sections were incubated with AEC for 14 min or DAB for 7 min (EnVision© System kit) to evoke, respectively, red or brown colour. Sections were washed with distilled water and counterstained with Mayers hematoxylin for 1 min and mounted with polyvinyl alcohol media pH 8.0. Two different anti-PRV rabbit sera, detecting respectively endosomal membrane penetration protein μ1C and cell attachment protein σ1, were used to identify PRV [14]. Additionally, immunohistochemistry was performed by antibodies against *Infectious pancreatic necrosis virus* (IPNV) [24] and SAV [13]. Pancreas samples from field outbreaks of IPN and pancreas disease (PD) in Atlantic salmon were used as positive controls for the latter two infections (provided by the Norwegian Veterinary Institute, Oslo). Heart samples from Atlantic salmon collected during a challenge study with HSMI were used as positive controls for PRV [20]. Negative controls were performed using 1% BSA instead of the primary antibody and by using rabbit serum collected prior to immunization.

RNA isolation

Total RNA was isolated from skeletal muscle and spleen samples stored in RNA*later* and from heparinized blood. Briefly, samples from spleen, white muscle (50 mg) and blood (15 μL) were homogenized in QIAzol Lysis Reagent (Qiagen, Hilden, Germany) using 5 mm steel beads and TissueLyser II (Qiagen) for 2 × 5 min at 25 Hz. Chloroform was added, samples were centrifuged and the aqueous phase collected. RNA was isolated using RNeasy Mini QIAcube Kit (Qiagen) according to manufacturer's instructions. RNA was quantified using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

RT-qPCR for viral RNA

Blood and skeletal muscle samples were screened for PRV by RT-qPCR using Qiagen OneStep kit (Qiagen) targeting PRV segment S1 as previously described [20]. A standard input of 100 ng (5 μL of 20 ng/μL) from the isolated total RNA was used in each reaction. The numbers of blood samples tested from each group were: A ($n = 25$), B ($n = 35$), E ($n = 20$), F ($n = 42$), G ($n = 42$) and H ($n = 10$), and for sample pairs of white skeletal muscle from changed and non-affected areas: Group A ($n = 9$), C ($n = 6$), E ($n = 14$), and non-affected white

skeletal muscle from groups without changes; i.e. Group F ($n = 6$) and G ($n = 6$).

Six spleen samples from each group, apart from Groups C (only muscle sampled) and D (only paraffin-embedded material), were analysed for other viruses and screened by RT-qPCR for IPNV, infectious salmon anemia virus (ISAV), piscine myocarditis virus (PMCV), SAV and viral hemorrhagic septicemia virus (VHSV). Reverse transcription was performed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with 1000 ng RNA input per sample. All qPCR analysis were run in duplicate in Mx3005P (Stratagene, La Jolla, CA, USA) using cDNA corresponding to 15 ng RNA input. The qPCR for detection of IPNV, ISAV, SAV and VSHV was performed with Taqman Universal PCR master mix (Applied Biosystems) including 300 nM primer and 200 nM probe in a 13 μL reaction volume. The following cycling conditions was used: 50 °C/2 min, 95 °C/10 min, 40 cycles of 95 °C/15 s, 58 °C/15 s and 60 °C/60 s. Elongation factor 1αβ (EF1αβ) was used as reference gene. For detection of PMCV, MESA Blue qPCR Mastermix Plus for SYBR assay (Eurogentec, Liège, Belgium) was used with primer concentration of 400 nM in a 15 μL reaction volume. The following cycling conditions was applied; 95 °C/5 min, 40 cycles of 95 °C/15 s and 54 °C/60 s. Primers and probes used in this study are listed in Table 2 [20,25-30]. The primers and probe used for SAV pick up all subtypes of SAV, including the subtypes currently found in Norway, i.e. SAV2 and SAV3.

The data was analysed by MxPro v4.10 (Stratagene). A sample was defined as positive if both parallel samples had a Ct < 35. The PRV RT-qPCR results from blood and skeletal muscle samples were used to calculate the mean Ct value and SD for each group. Ct value for samples were no Ct value was found was set to 35. Statistical analyses of differences were done by Wilcoxon matched pairs signed rank test using GraphPad Prism (GraphPad Software inc., USA), and p-values of $p \leq 0.05$ were considered as significant.

Results

Macroscopic examination

All fish of all groups were in normal condition and with no apparent external pathological changes. Following autopsy, large discoloured focal changes in the white muscle, mainly in the cranio-dorsal and abdominal regions, were found in Groups A, B, C, D and E, but not in Groups F, G and H, including fish kept in in-house tanks and in wild-caught individuals (Figure 1 and Table 3). One faintly pigmented focus, approximately 2 mm in diameter, was detected in one individual in Group F. This change would have gone unnoticed under normal abattoir conditions.

Table 2 Nucleotide sequences of primers and probes used in this study

Target	Primer/probe	Sequence (5′ → 3′)	Reference
PRV	S1 Fwd	TGCGTCCTGCGTATGGCACC	[20]
	S1 Rev	GGCTGGCATGCCCGAATAGCA	
	S1 Probe	ATCACAACGCCTACCT	
SAV	nsP1-Fwf	CCGGCCCTGAACCAGTT	[27]
	nsP1-Rev	GTAGCCAAGTGGGAGAAAGCT	
	nsP1-Probe	CTGGCCACCACTTCGA	
ILAV	S7 Fwd	TGGGATCATGTGTTTCCTGCTA	[30]
	S7 Rev	GAAAATCCATGTTCTCAGATGCAA	
	S7 Probe	CACATGACCCCTCGTC	
IPNV	VP3 Fwd	CGACCGACATGAACAAAATCA	[28]
	VP3 Rev	AGTTGCAGCTGTATTCGCACA	
	VP3 Probe	TCTAGCCAACAGTGTGTACGGCCTCCC	
VHSV	N Fwd	GACTCAACGGGACAGGAATGA	[29]
	N Rev	GGGCAATGCCCAAGTTGTT	
	N Probe	TGGGTTGTTCACCCAGGCCGC	
PMCV	Fwd	TTCCAAACAATTCGAGAAGCG	[25]
	Rev	ACCTGCCATTTTCCCCTCTT	
EF 1αβ	Fwd	TGCCCCTCCAGGATGTCTAC	[25]
	Rev	CACGGCCCACAGGTACTG	
	Probe	AAATCGGCGGTATTGG	

In general, each affected fillet had only one focal change, but occasionally there were multiple foci with varying degrees of discolouration within a single focal change. Foci extended from 1–3 cm and could involve 2–6 myotomes. Incisions through foci revealed extension of the discolouration deep into the muscle (Figures 1B and D). The changes generally appeared as either red or melanised (Figure 1 and Table 3). Two changes classified as red contained both red and melanised discolouration (Figure 2).

Histological examination

Red focal changes

Red focal changes in individuals of Groups A, C and E were all characterised by an acute haemorrhagic necrotic myositis. In more detail, we observed severely disorganised architecture of tissues including several myocytes with a complete loss of striation conformal with coagulation necrosis (Figure 3A). Organisation into granulomas was not observed. Poikilocytosis was highly prevalent, as both elongated and rounded erythrocytes were present. Focal areas of haemorrhage and accumulation of extravasal erythrocytes were present in all red changes accompanied by coagulation necrosis of myocytes. Erythrocytes and macrophage-like cells were observed both between and within necrotic myocytes (Figure 3B). In some changes, myocytes adjacent to the affected site showed

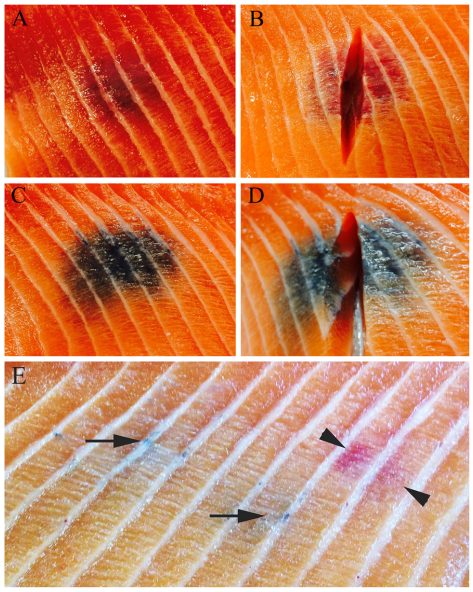

Figure 1 Gross pathological changes of white muscle. (A) A red focal change in the muscle of the craniodorsal region of the abdominal wall. **(B)** An incision through a red focal change showing discolouration extending deep into the fillet. **(C)** A melanised focal change detected in the same anatomical location in a different fish. **(D)** A melanised focal change with extensive amounts of a whitish tissue extending into the depth of the fillet. **(E)** A muscle fillet with two faint melanised lesions (arrows) and one red focal change (arrowheads).

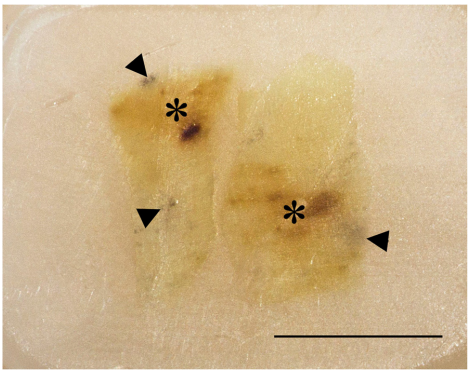

Figure 2 Gross image of paraffin-embedded red focal change. Transient form with both red (asterisk) and melanised (arrowheads) changes. Scale bar = 1 cm.

Table 3 Macroscopic distribution of red and melanised focal changes

Group	Fish (n)	Melanised changes (n)	Red changes (n)	No changes (n)	Heart changes* (n)
A	25	9	1	15	20
B	35	1	0	34	5
C	10	3	7	0	n/a
D	26	26	0	0	n/a
E	20	17	1	2	15
F	42	1	0	41	0
G	42	0	0	42	0
H	10	0	0	10	0

*Detection of focal or multi-focal endocarditis, myocarditis or epicarditis. (n/a - Not available).
The lesions in the white muscle were macroscopically classified as either red or melanised focal changes.

signs of regeneration displaying a basophilic appearance and a central rowing of nuclei. Occasional melano-macrophages were detected. Fibrosis was present to some degree, mainly associated with vacuoles that varied in size and shape (Figure 3C). Staining with Perls' Berlin blue technique confirmed the presence of haemosiderin in the periphery of the vacuoles, especially in association with large infiltrates of erythrocytes (Figure 3D).

In the two changes characterised as red with some visible melanisation (Figure 2), the histological picture concurred with the description above, whereas the findings in the melanised portion was consistent with the description of the melanised focal changes (below).

Melanised focal changes

Melanised focal changes in individuals of Groups A-F were characterised by chronic degenerative inflammation with well-organised granulomas. This included central severe fibrosis, but also peripheral acute extensive tissue destruction and necrosis. Melano-macrophages were abundant throughout the granulomatous tissue and within well-organised granulomas (Figure 4A). Vacuoles were observed throughout the affected areas (Figure 4B). Signs of repair with extensive fibrosis and regeneration were detected in the chronically inflamed lesions (Figure 4C), confirming previous tissue destruction and subsequent repair. Haemorrhages were not observed. Macrophages with hemosiderin were detected in association with melano-macrophages, though only sparse amounts of positive cells were observed (Figure 4D). Group F contained one individual with a very faint pigmented spot, and examination of this sample showed muscle necrosis and some inflammatory infiltrates containing melano-macrophages.

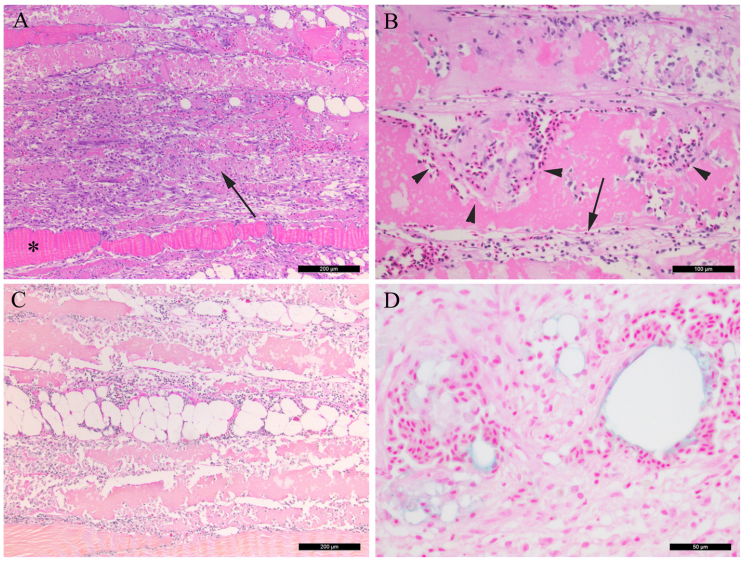

Figure 3 Histological investigations of red focal changes. (A) Necrotic myocytes display pale cytoplasm and appear homogenous without striations (arrow). Myocyte with striations (asterisk) (HE staining). **(B)** Necrotic myocytes with erythrocytes apparently located within the myocytes (arrowheads) and some infiltrations of macrophage-like cells (arrow) (HE staining). **(C)** Centrally located vacuoles of varying size, surrounded by moderate levels of collagen (intense red) and modest numbers of leukocytes (van Gieson staining). **(D)** Focal haemorrhage surrounding vacuoles. The edges of the vacuoles are positive for ferric iron (blue colour) (Perls' Berlin blue staining). **(A, C)** scale bar = 200 μm, **(B)** scale bar = 100 μm, **(D)** scale bar = 50 μm.

Hearts

In Groups A, B and E, moderate endocarditis, myocarditis (Figure 5) and epicarditis conformal with HSMI-related changes were detected (Table 3). Focal infiltrates of leukocytes were observed in either the compact or the spongious layers or both, and sometimes inflammatory foci were seen in the atrium. Degeneration of muscle fibres was not detected. No changes were observed in the fish kept in in-house tanks or in the wild fish (Groups F-H).

Immunohistochemistry for PRV

All focal white muscle changes in Group A-F were investigated and found positive for PRV-antigen. In general, the staining intensity was most prominent in acute compared to chronic stages, as assessed by the degree of organisation and content of connective tissue.

Red focal changes

Large amounts of PRV antigens were found in macrophage-like cells and in erythrocytes in the haemorrhagic necrotic tissue (Figure 6A). PRV-antigen positive cells were observed both outside and within necrotic myocytes. Myophagocytosis was highly prevalent, with substantial number of PRV-antigen positive macrophage-like cells within myocytes. Staining was cytoplasmic even in most PRV-antigen positive cells, though cells also displayed polar granular staining (Figure 6B). Such cytoplasmic PRV inclusions have previously been identified with confocal microscopy and immunofluorescent staining as viral factories [20].

Melanised focal changes

Immunohistochemistry from all affected groups, including the single individual in Group F, revealed intense reactivity against PRV in granulomas and in mononuclear cells (Figures 6C and D). Positive macrophage-like cells were scattered throughout the chronically inflamed areas. In PRV-antigen positive cells staining was observed predominantly as cytoplasmic inclusions (Figures 6E and F). Melano-macrophages positive for PRV were occasionally detected in necrotic myocytes (Figure 6G), but in general they were infiltrated with PRV-positive macrophage-like cells (Figure 6H). A macroscopically faint discolouration found in one individual revealed scattered necrotic myocytes containing PRV-antigen positive macrophage-like cells and occasional melano-macrophages. The two different anti-PRV sera, detecting respectively capsid protein μ1C and σ1, both had similar staining patterns.

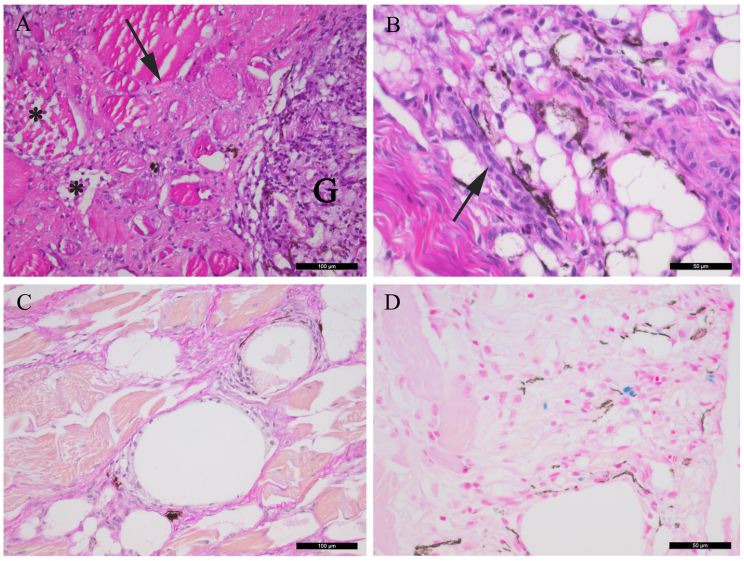

Figure 4 Histological investigations of melanised focal changes. (A) Transverse section of necrotic muscle cells (asterisk) and severe fibrosis (arrow) with infiltrates of leukocytes. A cell-rich granuloma with melanin-containing cells is present in the right part of the picture (G), displaying a heterogeneous morphology (HE staining). **(B)** Severe vacuolisation and adipocytes with surrounding melano-macrophages (black) and clusters of regenerating myocytes with a basophilc cytoplasm (arrow) (HE staining). **(C)** Multiple vacuoles in an area with severe fibrosis (intense red staining) (van Gieson staining). **(D)** Several iron-containing macrophage-like cells (blue staining) in association with melano-macrophages (black) (Pearls' Berlin blue staining). (A, C) scale bar = 100 μm, (B, D) scale bar = 50 μm.

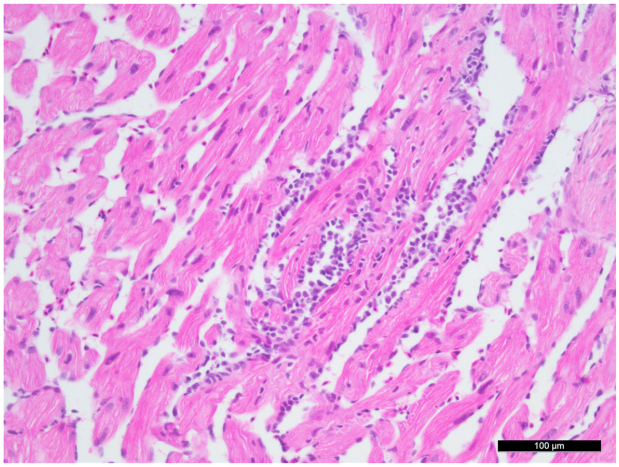

Figure 5 Histological image of cardiac spongious layer. Infiltrates of leukocytes are visible in endocard and myocard. Scale bar = 100 μm.

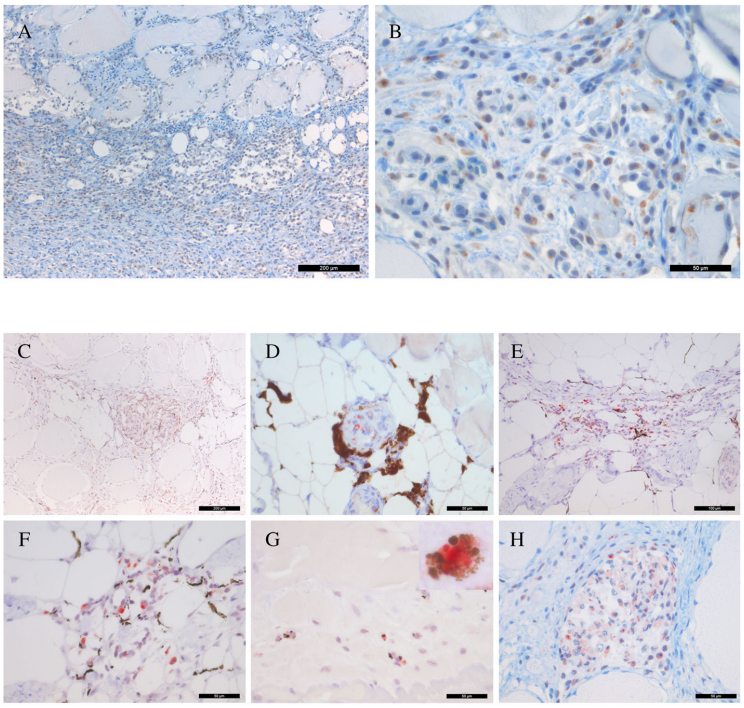

Figure 6 Immunohistochemical staining for PRV antigens of red (A-B) and melanised (C-H) focal changes. (A) Abundant amounts of PRV-positive cells (brown) and erythrocytes in the necrotic muscle tissue. **(B)** Transverse section of necrotic myocytes with intracellular PRV-positive macrophage-like cells and erythrocytes (brown). Distinct granular staining is present in the cytoplasm of the macrophage-like cells. **(C)** A well-organised granuloma is present in the center of the image, heavily positive for PRV (red), and with vast amounts of elongated melano-macrophages (black). **(D)** A single granuloma with PRV-positive cells (red) surrounded by heavily pigmented melano-macrophages. **(E)** A focus with necrotic tissue and infiltrates of melano-macrophages (black) and macrophage-like cells positive for PRV (red). **(F)** A close-up of E where the distinct reaction in the cytoplasm is evident (red). **(G)** PRV antigen (red) in melano-macrophages (black) in a necrotic myocyte. Higher resolution image in the upper right corner (100 x). **(H)** A single necrotic myocyte undergoing phagocytosis and containing abundant PRV-positive macrophage-like cells (red). (A, C) scale bar = 200 μm, (E) scale bar = 100 μm, (B, D, F, G, H) scale bar = 50 μm

Controls

In the PRV- RT-qPCR negative control group (Group H), no changes or PRV staining were detected. Control white-muscle samples from fish with red or melanised focal changes were all PRV-antigen negative unless necrosis was present. When necrosis was present, i.e. without macroscopically red or melanised changes, the myocytes contained PRV-antigen-positive macrophage-like cells. A similar occurrence of positive cells in degenerated muscle cells was also seen in occasional fish without focal white muscle changes. No staining was detected when the primary antiserum was omitted or replaced with the pre-immune serum. Six fish from each group were further investigated for the presence of IPNV [24] and SAV [13] with immunohistochemistry. All fish were negative.

PRV detection by RT-qPCR

PRV load in blood

The PRV loads in blood samples of Groups A, B, E-H were assessed by RT-qPCR (Figure 7A). All groups in which melanised changes were found (Groups A, B, E) contained RT-qPCR PRV positive fish. High PRV loads were detected in Group A (Ct 20.7 ± 0.8) and Group E (Ct 20.7 ± 1.4), in which prevalences of melanised changes were 40% and 90%, respectively. The viral load was much lower in Group B (Ct 32.8 ± 1.7) where melanised changes were much less prevalent (3%). High levels of PRV were also detected in Group F (Ct 20.6 ± 1.2) and Group G (Ct 21.4 ± 1.4) where melanised focal changes were absent. The wild fish of Group H were the only group negative for both PRV and melanised changes.

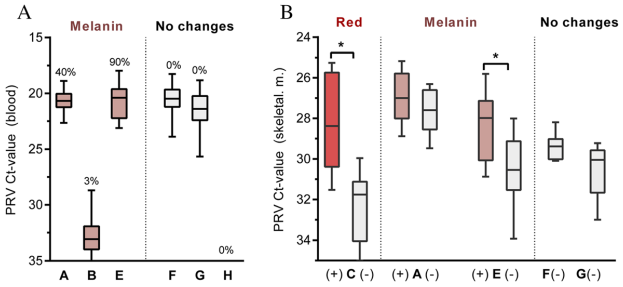

Figure 7 RT-qPCR analysis for PRV in blood (A) and white skeletal muscle (B). Fish groups with melanised, red or no changes are coloured brown, red, and grey, respectively. (A) Detection in blood presented by box plot with whiskers indicating max/min of the Ct-values from Group A ($n = 25$), B ($n = 35$), E ($n = 20$), F ($n = 42$), G ($n = 43$) and H ($n = 10$). The percent of fish in each group with melanised focal changes are displayed. (B) Samples from red or melanised focal changes (+) and non-affected areas (–) from the same individual. Box plot and whiskers showing max/min of the Ct-values from Group C with red focal changes ($n = 6$), and from Groups A ($n = 9$) and E ($n = 14$) with melanised focal changes. Only non-affected tissue (–) could be tested from groups without changes; i.e. Groups F ($n = 6$) and G ($n = 6$). Analyses were performed using Wilcoxon matched pairs signed rank test. *$p < 0.05$.

Overall, the load of PRV in blood was not correlated with the presence of melanised changes.

PRV load in red and melanised changes

The PRV loads in samples from red and melanised changes were assessed by RT-qPCR and compared to samples collected from non-affected white muscle tissue of the same individual fish (Figure 7B). The red changes, observed in Group C, contained a significantly higher viral load compared to control tissue with Ct values 28.2 ± 2.4 and 32.3 ± 1.8, respectively ($p < 0.05$). The viral loads in melanised changes from Group E were also significantly higher than that of the control (Ct 28.3 ± 1.5 and Ct 30.4 ± 1.6) ($p < 0.05$), while no significant difference was found from group A. White muscle tissues from fish with no spots in Groups F and G had a Ct value of 29.4 ± 0.7 and 30.5 ± 1.4, respectively. The viral loads observed in the white muscle tissues (Figure 7B) were significantly lower than that observed in blood (Figure 7A).

Controls

The six spleen samples from each of Group A, B, E-H were all negative for IPNV, ISAV, PMCV, SAV and VHSV by RT-qPCR.

Discussion

In this study, we investigated red and melanised focal changes as well as transient, partly red and melanised changes in white muscle of Atlantic salmon. Histological investigations revealed that red focal changes were dominated by haemorrhages and myocyte necrosis, consistant with acute manifestations of muscle damage. The focal melanised changes were dominated by granulomatous tissue rich in collagen, indicating chronic inflammatory response. In these changes, melano-macrophages were abundant, and iron-containing macrophage-like cells were observed, indicating previous haemorrhage. The transient forms displayed both red (acute) and melanised (chronic) changes as described above. Macroscopic observations showed that all forms may occur in the same individual simultaneously. Together, these results indicate that the melanised focal changes arise as a consequence of chronic inflammation.

The common denominator to all red and melanised changes was the presence of large amounts of PRV antigens detected by immunohistochemistry. In red focal changes, i.e. acute manifestations, PRV antigen was found in erythrocytes and macrophage-like cells in necrotic myocytes. In addition, the viral loads as measured by RT-qPCR, were significantly higher in red focal changes than in surrounding non-affected muscle. In melanised changes, i.e. chronic changes, PRV-antigen was detected in macrophage-like cells and melano-macrophages within granulomas and in less organised granulomatous tissue. Our findings indicate that a focal PRV infection is a premise for the transition from red to melanised focal changes, and the lack of capability to eliminate PRV or PRV-antigen is the driving power behind this process.

The results of immunohistochemistry clearly linked PRV to the focal lesions. Several approaches unanimously demonstrated the specificity of the PRV immunostaining. Two different PRV rabbit antisera both displayed cytoplasmic staining of macrophage-like cells within the white muscle changes. Samples from areas without red and melanised focal changes from affected or non-affected fish were in most cases negative, but macrophage-like cells in solitary degenerated myocytes were positive. No other viruses were detected in any of the groups. One group of wild salmon from a river located far away from commercial farming was included in the study (Group H). The fish

were found to be PRV negative in blood by RT-qPCR, negative for melanised focal changes by visual examination, and PRV-antigen negative by immunohistochemistry. As both PRV and melanised focal changes are prevalent in farmed Atlantic salmon, it was imperative for this study to include such negative control material in the investigations.

Erythrocytes are important target cells for PRV [19,21]. In our study positive staining for PRV was also found in macrophage-like cells and melano-macrophages. We do not know if PRV replicates in all these different cell types. Replication of PRV takes place in cytoplasmic structures called viral factories, which appear as dense inclusions [20]. Intracellular structures with dense staining that may resemble viral factories were prevalent findings in the red and melanised focal changes. This is in line with an on-site propagation of PRV. The amount of PRV RNA, as assessed by Ct values from red focal changes was significantly higher than from corresponding non-affected muscle tissues from the identical individual. This correlation was not fully consistent for melanised changes. This indicates that replication of PRV occurs at the sites with red changes, while PRV-antigen persists into the formation of granuloma. Although macrophages engulf viruses to inactivate them, many viruses are able to replicate in macrophages [31-33]. This may provide a long-term shielded environment for viral replication, and the process will attract more macrophages and culminate in a chronic inflammatory response. Comparatively, avian orthoreoviruses (ARV) viral antigen and RNA have been documented in macrophages [34]. ARV are ubiquitous in avian farming and belong to the same genus as PRV, and virulent ARV strains have shown enhanced ability to replicate in macrophages [35].

PRV replicates in erythrocytes and therefore virus loads were assessed in blood samples. All groups with focal muscle changes were positive for PRV by RT-qPCR in blood. However, similar viral loads were found in groups without changes. The general PRV load in a fish, as mirrored by the amount in blood, is thus not predicative for the presence of focal muscle changes. This indicates that PRV infection by itself is not sufficient to induce melanised focal changes in the white muscle and that environmental and management factors may be of importance. We cannot exclude that the condition may be initiated by for example trauma. However, melanisation is an insignificant problem in rainbow trout farmed under similar conditions with similar handling and risk of trauma. It could be mentioned that also HSMI, which is prevalent in farmed Atlantic salmon, is negligible in farmed rainbow trout.

High loads of PRV in blood were detected by RT-qPCR in land-based Groups F and G where the fish were almost devoid of macroscopically observable muscular changes. The average weight of the fish in these groups was approximately 2.8 kg while that of the other groups of farmed fish were 4.4 kg. It should not be ruled out that development of the changes is more frequent late in the production cycle. Another possibility is that the PRV strain in Groups F and G was less virulent or the strain of fish could be less susceptible, but this is not supported by the fact that the fish was kept and raised at the same farm as Group E. The almost complete lack of melanisation in Groups F and G indicates that the difference in occurrence of melanisation was related to factors in the management of the populations. In a previous study, genetic constitution, vaccination status and smoltification regime were addressed with respect to the frequency of melainised focal changes [10]. Higher frequency was detected in triploid versus diploid fish and in fish smoltified at elevated temperatures after vaccination. The lack of focal muscle changes in the land-based Groups F and G, vaccinated or non-vaccinated, supports previous findings suggesting that vaccination is not a causative factor in the pathogenesis [10-12].

In the present study, the red and melanised focal changes were observed in white muscle. Classically, the muscle lesions related to HSMI are detected in the red muscle and myocardium. However, degeneration and inflammation in white muscle have also been reported in fish with HSMI [16]. HSMI is mainly found during the first months after sea transfer. In contrast, melanised focal changes are predominantly observed at slaughter, indicating a long-term sub-clinical inflammation. The knowledge on long-term effects of a PRV-infection on white muscle is limited. HSMI and focal melanised changes should be considered as two separate conditions; both associated with PRV, but do not necessarily appear simultaneously.

Large amounts of PRV antigens were found in all red and melanised focal changes in salmon groups widely distributed both in time and in geographical locations. The samples from aquaculture used in this study originated from different farm sites west and north on the Norwegian coast, including areas in which SAV infection or the associated Pancreas disease have not been reported. This suits well with the involvement of a ubiquitous aetiological agent such as PRV. Furthermore, the problem with melanised focal changes containing PRV has existed for many years, as demonstrated by the positive results in the archived material (Group D).

Melaninsation contributes to encapsulation and prevents dissemination of intruding pathogens, this being a prominent immune response in arthropods. Such responses therefore have functions similar to those of granulomas in vertebrates. Melanisation possibly also aids the healing process in arthropods. Reactive oxygen species generated during melanisation is thought to be toxic to pathogens, but also to the arthropod host, causing a strict localisation of melanin to the site of inflammation [36].

Our understanding of the function of melanin in the piscine immune responses is in its infancy. Chronic inflammation in fish may appear with abundant pigmentation, as has been found in the peritoneal cavity after deposition of oil-adjuvanted vaccines [1,8]. In our study virus antigen was found at sites with granulomatous inflammation, sometimes encapsulated in well-advanced granulomas.

Our findings indicate that PRV is a premise for the progression of red to the chronic melanised focal changes; however, it does not establish that PRV initiates the process leading to the formation of red focal changes. When a focal PRV infection is established, either as an infection of the myocytes or through infiltrating erythrocytes or macrophage-like cells, the immune response is not able to eliminate the infection to stop the formation of granuloma and melanisation. Myocytes may have been virus-infected prior to necrosis; however, we have no indication for this assumption. Haemorrhage may have developed as a consequence of myocytic necrosis. PRV antigen was found within encapsulated granuloma, the latter being the hallmark of an immune response where the host immobilises and walls off a persistent intruder. Melano-macrophages are known for their participation in such reactions in fish and their presence accounts for the discolouration found in the melanised focal changes. As no melanised focal changes were identified that were devoid of PRV antigen, we believe this presence to be the premise for the prolonged and non-dissolving granulomatous reaction seen in melanised focal changes.

In conclusion, the transition from acute red to chronic melanised changes was justified. Both red and melanised focal changes contain large amounts of PRV antigens, however, environmental and management factors may be pivotal for the initial development of these changes. We suggest PRV to be associated with red and melanised focal white-muscle changes in Atlantic salmon.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
HB: Planning, sampling material from groups F and G, writing manuscript and histological experiments and analysis; ØW: PCR experiments and analysis and writing manuscript; PGF and TH: Experimental design and sampling of groups E, F and G; HS: Organising sampling from the Lerøy groups; HRS: sampling from groups B and C, KBE and EM: sampling from group A; AK: histology analysis; ER: Planning, PCR analysis and writing manuscript; EOK: Planning, sampling material from groups D, F, G and H, histological analysis, writing manuscript and co-ordinating the overall work. All authors commented on the manuscript.

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II

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ORIGINAL ARTICLE

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Melanized focal changes in skeletal muscle in farmed Atlantic salmon after natural infection with *Piscine orthoreovirus* (PRV)

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Abstract

Melanized focal changes in skeletal muscle of farmed Atlantic salmon (*Salmo salar*) are a major quality problem. The aetiology is unknown, but infection with *Piscine orthoreovirus* (PRV) has been associated with the condition. Here, we addressed the pathogenesis of red and melanized focal changes and their association with PRV. First, a population of farmed fish (PRV-negative prior to sea transfer) was sequentially investigated throughout the seawater period. The fish were autopsied and tested for PRV infection. Muscular changes were described by macroscopy and histology, and a classification system was established. Second, in an experimental infection trial, PRV was injected intramuscularly to induce changes. The farmed fish was gradually infected with PRV. Red focal changes occurred throughout the observation period with a low prevalence regardless of PRV status. Melanized changes were highly diverse and their prevalence increased during the trial. Changes of low macroscopic grade and histological category were more prevalent in PRV-negative fish. Diffuse granulomatous melanized changes only occurred after PRV infection. No muscular changes were observed in the experimentally challenged fish. Our studies do not indicate that PRV infection causes red focal changes, but seems important in the development of granulomatous melanized changes.

KEYWORDS
black spot, inflammation, melanin, melano-macrophage, red spot

1 | INTRODUCTION

Melanized focal changes ("black spots") in skeletal muscle (fillet) are currently the most serious quality problem in Atlantic salmon (*Salmo salar*) farming in Norway, with an estimated presence in about 20% of the fillets at slaughter (Mørkøre et al., 2015). Morphological investigations revealed chronic focal inflammation with melano-macrophages causing the black/brown colour (Koppang, Haugarvoll, Hordvik, Aune, & Poppe, 2005; Larsen et al., 2012). The changes display the characteristic features of chronic focal necrotizing granulomatous myositis with large extracellular fat vacuoles. These changes were originally attributed to unwarranted vaccine side effects (Koppang et al., 2005), but were later observed in both vaccinated and non-vaccinated fish (Berg, Yurtseva, Hansen, Lajus, & Fjelldal, 2012; Larsen et al.,2014). In all studies on melanized focal changes, samples have been collected at the end of the production cycle, observed as well-advanced, chronic, melanized changes exposed after filleting.

The industry has suggested a correlation between red (also known as "bleedings") and melanized focal changes. Compared with the latter, red changes seem rare, but their prevalence is unknown. Presumably, the red changes are located at the same site as the melanized focal changes, which are most frequent in the cranio-ventral part of the abdominal musculature (Mørkøre et al., 2015). Bjørgen et al. (2015) investigated both red and melanized focal changes at slaughter for presence of PRV using both immunohistochemistry (IHC) and RT-qPCR. All samples proved PRV-positive by PCR; however, the virus is ubiquitous in farmed salmon some months after their sea transfer, and as it replicates in erythrocytes (Finstad et al., 2014), the virus can be found in all organs. Immunohistochemistry demonstrated presence of PRV in macrophage-like cells and in erythrocytes in red focal changes, and in inflamed melanized tissue including even the centre of melanized granulomas. Importantly, transient forms between red and melanized focal changes were also characterized, confirming a link between the two manifestations. Based on their results, Bjørgen et al. (2015) concluded that PRV was a premise for the development of melanized focal changes. Subsequently, these results were challenged by Krasnov, Moghadam, Larsson, Afanasyev, and Mørkøre (2016), suggesting a bacterial component to the pathogenesis, this being based on the detection of transcripts of various prokaryotic rRNA in melanized changes. None of these studies concluded on an initial cause of the condition. The cause of intramuscular bleedings and focal melanization thus remains unknown.

Here, we investigate the sequential development of red and melanized focal muscle changes in a commercial fish population over a period of 15 months. The aim was to reveal the prevalence, severity and anatomical location of red and melanized changes throughout a production period in a seawater farm. Additionally, the melanized changes were systematically classified based on histological differences. We investigated the focal changes for different pathogens with emphasis on PRV, using RT-qPCR, IHC and *in situ* hybridization (ISH). In addition, we conducted an *in vivo* experiment trying to induce red and melanized focal changes by injecting PRV in Atlantic salmon. Our studies provide novel insight into the development and nature of a serious quality problem in Atlantic salmon production.

2 | MATERIALS AND METHODS

2.1 | Field trial

2.1.1 | Fish and sampling information

A total of 240 000 Atlantic salmon, Mowi strain, were transferred to sea water at Mowi's location at Svåsand, Hardanger, Norway, in autumn 2015 with an average weight of 110 g. The fish were intraperitoneally vaccinated according to national requirements. Prior to sea transfer, head-kidney samples from the fish were tested for presence of PRV by RT-qPCR (N = 53) by PatoGen Analyse, Ålesund, Norway. From autumn 2015 to December 2016, seven major samplings were conducted (I–VII): two in 2015 and five in 2016 (Table 1). At least 600 individuals were autopsied at each time point. In order to thoroughly follow the PRV status of the fish, six minor samplings were conducted in addition to the seven main samplings (E1–E6).

In all of the main samplings, the collected samples included peripheral blood (in heparin) and gill, spleen and muscle samples (on RNAlater) from the first 60 individuals. These samples were investigated for PRV-1 by RT-qPCR. In this work, PRV equals PRV-1. Between 27 and 40 fish were at each main sampling selected for

TABLE 1 Main samplings I–VII and minor samplings E1–E6

Name/date for sampling	Weeks post-sea transfer	Number of fish	Average size kg/cm	Selected fish	Sea temperature (°C) (at 0.5 m)	Sea temperature (°C) (at 5 m)	Sea temperature (°C) (at 15 m)
E1 28/09/15	2	59	n.m.	–	12.7	13.7	14.2
I 26/10/15	4	606	0.11/22.1	36	11.0	11.2	11.5
E2 30/11/15	8	60	n.m.	–	6.9	n.a.	n.a.
II 22/12/15	11	620	0.31/30.0	36	7.1	7.7	7.7
E3 19/01/16	15	61	n.m.	–	6.8	6.9	6.9
III 15/02/16	19	608	0.49/35.5	40	3.6	4.8	4.8
E4 15/03/16	23	61	n.m.	–	4..9	6.0	6.0
IV 18/04/16	27	610	0.75/40.8	27	7.3	6.2	6.2
E5 23/05/16	32	60	n.m.	–	12.0	8.3	8.3
V 20/06/16	36	610	1.24/48.7	30	15.3	11.1	11.1
E6 26/07/16	41	63	n.m.	–	18.0	12.0	12.0
VI 13/09/16	48	616	2.9/61.3	33	15.9	15.3	15.3
VII 05/12/16	59	633	4.9/68.7	31	4.9	6.3	6.3

Date, weeks post-sea transfer, number of fish autopsied, average size and number of selected fish for histology at each time point. Sea temperature at different depths at sampling dates is also included. All types of samples were obtained in I–VII from selected fish, and only blood was sampled in E1–E6. n.m. = not measured.

a closer investigation of macroscopic changes and for histological analyses. Representative numbers of the different macroscopic manifestations present at each sampling were selected for in-depth qualitative analysis, including between 5 and 8 control fish (fish with no macroscopic abnormalities). From these selected fish, affected muscle and corresponding unaffected muscle were collected on both formalin and RNAlater. Liver, spleen, head kidney, heart and the sideline containing skin and red and white muscle were collected on formalin. In the additional minor samplings, blood from at least 60 randomly selected fish was collected. All RT-qPCR analyses were performed by PatoGen Analyse.

2.1.2 | Macroscopic registrations

After filleting, the changes were registered according to anatomical location (Figure 1), type and grade of change (Figure 2). The grade of both red and melanized changes was assessed according to the scoring system used by Mowi, ranging from grade 1 to grade 3, where 1 was very faint discoloration, 2 was a distinct but not severe discoloration and 3 was a prominent and severe discoloration. Grade 1 would probably not have been registered as a quality abnormality at slaughter by some producers, whereas grades 2 and 3 would have implied a quality reduction of the fillet. Representative examples are given in Figure 2. Statistical calculations on the macroscopic registrations were performed with GraphPad Prism©, using Fisher's exact test to calculate the association between sampling groups.

2.1.3 | Histology—standard and special stains

The formalin-fixed samples were dehydrated through graded ethanol baths, cleared in xylene and embedded in paraffin. Sections were made from all collected samples (all organs) and were subsequent to rehydration and stained with haematoxylin and eosin (HE staining). Three red and melanized changes from each sampling were subjected to Gram, Giemsa and periodic acid–Schiff (PAS) staining. Additionally, five active granulomas (grade 9) were stained for mycobacteria (Ziehl–Neelsen's method). All procedures were performed in accordance with Bancroft and Gamble (2008).

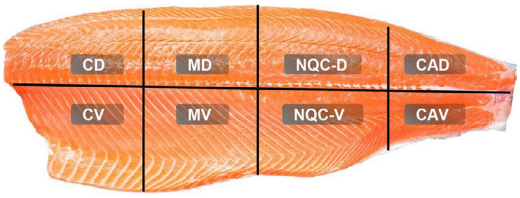

FIGURE 1 Epaxial and hypaxial muscle ("fillet") regions referred to by regions in registration of prevalence and description of muscle changes. The fillet is divided into four parts of equal length in the cranio-caudal direction and further in epaxial and hypaxial parts located dorsal and ventral, respectively, to the lateral line. CD/CV—cranio-dorsal/cranio-ventral; MD/MV—mid-dorsal/mid-ventral; NQC-D/V—Norwegian Quality Cut dorsal/ventral; CA-D/V—caudodorsal/ventral [Colour figure can be viewed at wileyonlinelibrary.com]

2.1.4 | IHC and ISH

Representative changes from each histological category were investigated with IHC for PRV as described previously (Bjørgen et al., 2015), using an antibody targeting the PRV σ1 protein. Briefly, sections were rehydrated and autoclaved. Inhibition was done with phenylhydrazine and blocking with goat normal serum diluted in 5% BSA/TBS (bovine serum albumin/Tris-buffered saline). The primary antibody was diluted in 1% BSA/TBS (dilution 1:700) and incubated for 30 min at room temperature. The sections were further incubated with an anti-mouse secondary antibody (Dako EnVision kit) and developed with AEC to evoke colour (red). PBS was used to wash between each step.

To detect viral RNA in the melanized changes, the slides underwent hybridization with RNAscope probe against a portion of PRV-1 genome segment L1 coding for PRV core shell (Advanced Cell Diagnostics, catalogue #705151). The method was performed using RNAscope (RED) (Advanced Cell Diagnostics, Newark, CA, USA) according to the manufacturer's instructions. The method has previously been used to detect PRV RNA in Atlantic salmon Di Cicco et al., 2018). A heart section from PRV-negative fish from a previously published challenge study was also included as a negative control (Finstad, 2014). From the same trial, a heart section from a PRV-positive fish with severe epicarditis was used as a positive control. RNAscope probe against the bacterial gene dapB (#701021) was used as a negative control on duplicates of the same sections to confirm absence of background and/or non-specific cross-reactivity of the assay.

2.2 | Experimental challenge study

A challenge experiment was performed at VESO Vikan aquatic research facility (Vikan, Norway). The Norwegian Food Safety Authority (NFSA), in accordance with the European Union Directive 2010/63/EU, approved the experiment. The experiment included 384 unvaccinated seawater-adapted Atlantic salmon, SalmoBreed strain. The fish were fed standard commercial food, kept in particle-filtered and UV-treated sea water at 12°C and 34 ‰ salinity, anaesthetized by bath immersion in benzocaine chloride (2–5 min, 0.5 g/10 L) prior to handling and killed with an overdose of benzocaine chloride (1 g/5 L). No fish were positive in a PCR screening for selected salmon pathogens prior to the initiation of the experiment.

In Tank 1, Group 1 was injected intraperitoneally (i.p.) with pelleted blood cells containing high loads of PRV (Lund et al., 2016), Group 2 was injected intramuscularly (i.m.) with purified PRV (Lund, 2016), and Groups 3 and 4 were controls given PBS and no treatment, respectively. In Tank 2, Groups 5–7 were given i.p. PRV-infected blood cells, purified PRV and Renibacterium salmoninarum, respectively, that all had been heat-inactivated at 85°C for 25 min. In Tank 2, Groups 8 and 9 were controls given PBS and no treatment,

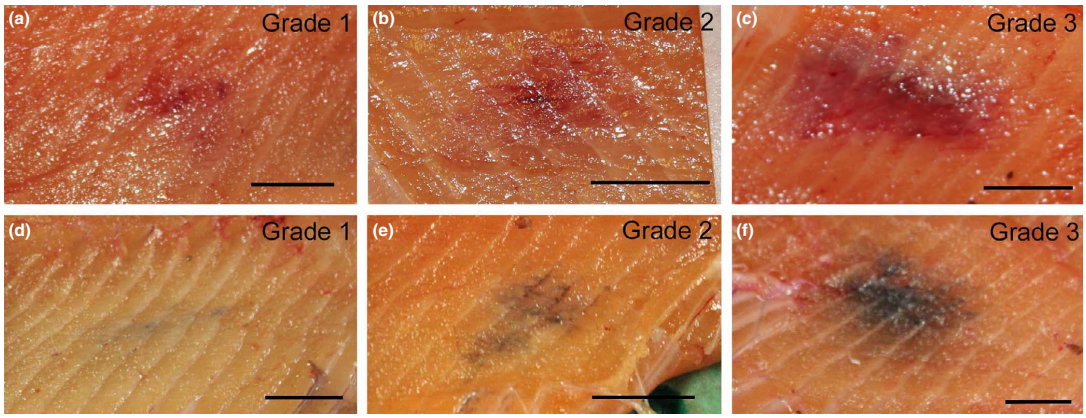

FIGURE 2 Macroscopic grading of red (a–c) and melanized (d–f) focal changes. Muscle changes were assessed as grade 1, 2 or 3 following the classification system as used by Mowi. Representative pictures from Sampling IV. Grade 1: small in size and/or weak in colour. Grade 2: small, but intense in colour, and/or larger in size. Grade 3: strong colour and large size. Scale = 0.9 cm [Colour figure can be viewed at wileyonlinelibrary.com]

respectively. The fish were observed for 18 weeks post-challenge (wpc), and six fish of each group were sampled at 3-week intervals. At each sampling, heparinized blood, spleen, kidney, heart and skeletal muscle tissues were collected in RNAlater and in formalin. The fish were filleted and white muscle examined visually for red or melanized changes. RNA was isolated and RT-qPCR for PRV was performed as described previously (Wessel et al., 2017).

3 | RESULTS

3.1 | Field study

3.1.1 | PRV detection by RT-qPCR

PRV was detected neither in head kidney from 53 fish prior to sea transfer nor in blood from fish from the first three main samplings (I–III) after transfer, that is, during a period of 19 weeks in sea water. Thereafter, the prevalence increased from 10% (6/60 fish) in Sampling IV (Ct values between 14.2 and 35.5), to 67% (41/61 fish) in Sampling V (Ct values between 17.0 and 35.8) to 98% (59/60 fish) in Sampling VI (Ct values between 15.5 and 31.7) (Figure 3).

In addition to blood, at least 30 gill, spleen and muscle samples were analysed from each sampling. PRV was detected in the gills from three fish in Sampling II (Ct values between 36.5 and 36.8), two fish in Sampling III (Ct values between 36.4 and 36.7) and 21 fish in Sampling IV. Spleen and muscle samples remained negative until Sampling IV.

3.1.2 | Macroscopic registrations

Both red and melanized focal changes occurred in all samplings (I–VII). Ninety-two per cent of these changes were in the cranio-ventral and mid-ventral regions of the fillet. Red focal changes macroscopically graded 1 to 3 occurred in all samplings. Their prevalence and severity remained stable throughout the observation period with a prevalence of about 4% for each sampling (Figure 4). The melanized focal changes first appeared with low frequency and were macroscopically graded as limited severity (grades 1–2). At later samplings, melanized focal changes became more frequent and their macroscopic severity increased (Figure 4). From Sampling V onwards, melanized focal changes graded 3 were observed (Figure 4 and Table 2).

A statistically significant increase in the prevalence of melanized focal changes was observed from Sampling I to Sampling II (from 6% to 12%). The prevalence was lower in Sampling III (11%) (not statistically significant, $p > 0.05$).

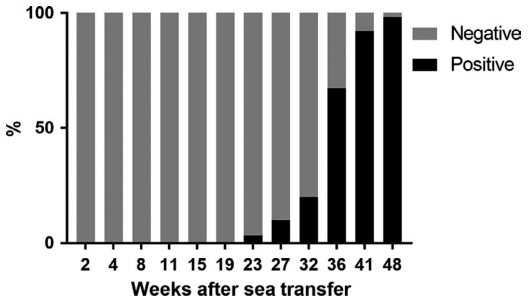

FIGURE 3 Percentage of PRV-negative versus PRV-positive farmed fish tested in peripheral blood by RT-qPCR. Fifty-nine to sixty-three fish were randomly selected in each sampling and analysed for PRV. The bars show that the percentage of PCR-positive fish increased steadily from detection. The Ct values in PCR-positive fish varied substantially, from 14.5 up towards cut-off value. Most Ct values in positive fish were in the range 20–30. Blood samples were not analysed (n.a.) in fish from Sampling VII (59 weeks after sea transfer)

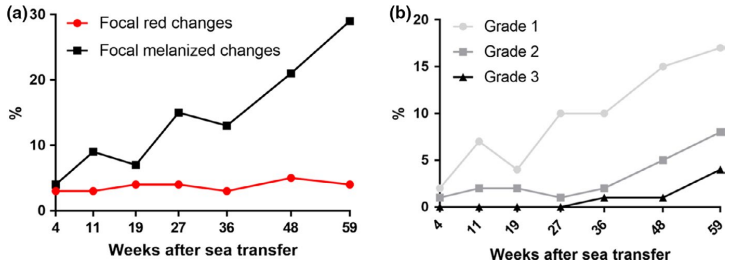

FIGURE 4 Graphs showing the prevalence of (a) red and melanized focal changes and (b) different melanized macroscopic grades at each sampling shown as weeks after sea transfer. (a) Prevalence of red (red line) and melanized focal changes (black line) in autopsied fish from Samplings I–VII, independent of macroscopic grade. (b) Prevalence of grade 1–3 melanized changes in fish from Samplings I–VII [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Macroscopic grades and histological categories of melanized focal changes present at each sampling

Main samplings	Weeks post-sea transfer	Macroscopic grades observed	Histological categories observed
I	4	I, II	1, 2, 4, 5
II	11	I, II	1, 2, 5
III	19	I, II	1, 2, 3, 4, 5
IV	27	I, II	4, 5
V	36	I, II, III	1, 2, 5, 6, 8, 9
VI	48	I, II, III	1, 4, 5, 9
VII	59	I, II, III	2, 4, 5, 7, 8, 9

The numbers show which categories and which macroscopic grades were present at each of the main samplings (I–VII).

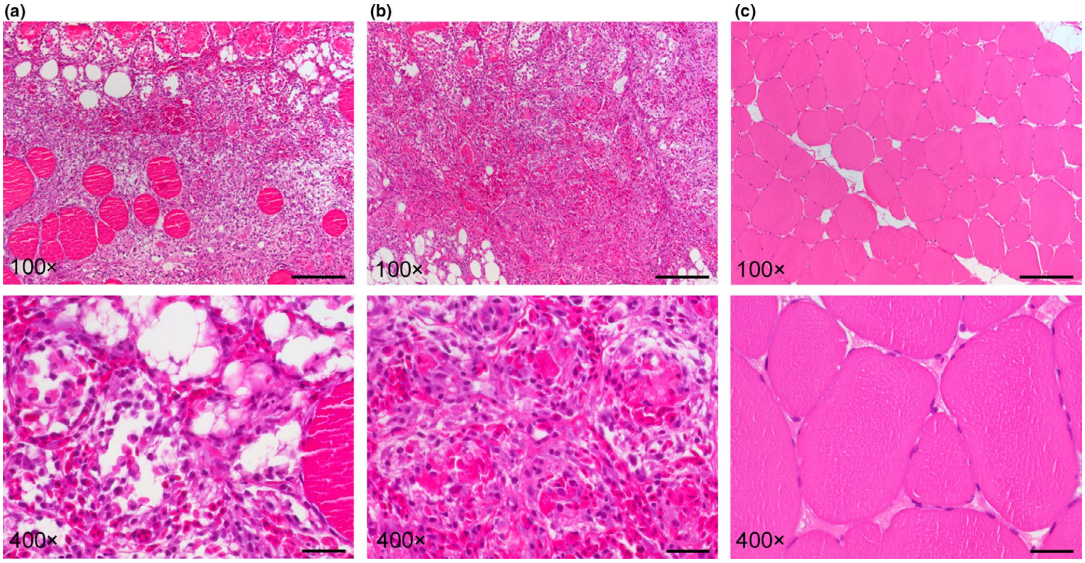

FIGURE 5 Histopathological characteristics of red focal changes (100X and 400X of the same image). (a) Myocyte necrosis with endo- and perimysial haemorrhage. Scattered intact myocytes and vacuoles are present. The 400X image shows endomysial haemorrhage and vacuoles (negative imprint of lipids). Inflammatory cells and activated fibroblasts are present. (b) Severe bleeding and infiltration of inflammatory cells. There is total loss of tissue architecture. Vacuoles of varying size are present in the lower corners. 400X shows endomysial haemorrhage and activated fibroblasts. (c) Control muscle from the same anatomical location shows intact myocytes of various sizes. Adipocytes are limited to the myosepta and occasionally to the endomysium [Colour figure can be viewed at wileyonlinelibrary.com]

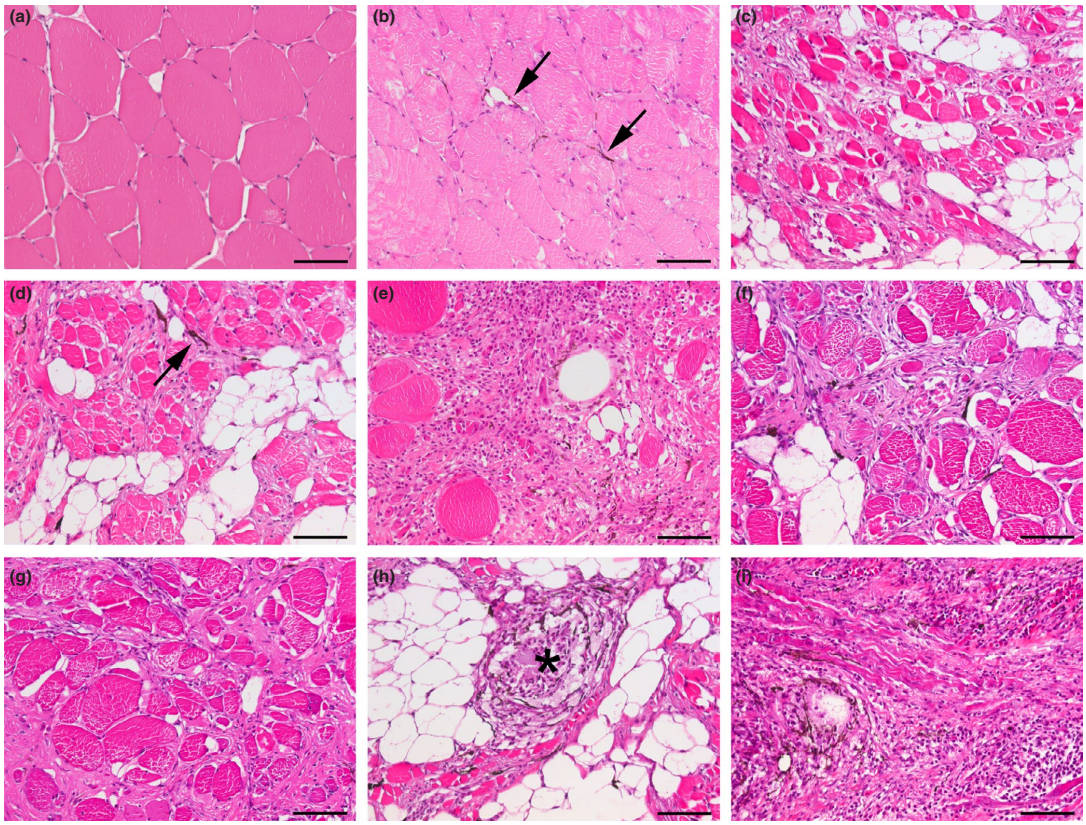

FIGURE 6 Examples illustrating the nine different histological categories used to classify melanized focal changes. (a) Category 1: no observable changes. Myocytes have a rounded-to-hexagonal shape (in cross section), varying sizes and peripheral nuclei. (b) Category 2: interstitial inflammation with endomysial presence of melano-macrophages that stretch out between (apparently) intact myocytes' surfaces (arrows). (c) Category 3: fibrosis between (apparently) intact myocytes and adipocytes. No melano-macrophages are observed. (d) Category 4: area with fibrosis and melano-macrophages (arrow) between apparently intact myocytes. (e) Category 5: area dominated by fibrosis and inflammatory cells. (f) Category 6: fibrosis and infiltrates of inflammatory cells including melano-macrophages. (g) Category 7: fibrosis without presence of melano-macrophages. (h) Category 8: focal granulomatous inflammation. Asterisk indicates the centre of a granuloma. (i) Category 9: diffuse granulomatous inflammation replacing normal tissue in large area. Scale bars = 50 μm [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Histological examination

3.2.1 | Red focal changes

Red focal changes from all samplings and with macroscopic grades 1, 2 and 3 displayed tissue changes with some variation, which seemed unrelated to the macroscopic appearance, as all variations described here were in all macroscopic grades. The histological findings varied from severe muscle necrosis with sparse haemorrhage to severe bleedings and severe necrosis. Haemorrhage was both endo- and perimysial in all changes. Some changes had scattered intact myocytes among macrophage-like cells and adipocytes (Figure 5a). Other changes were totally devoid of normal tissue architecture occurring with endomysial haemorrhage, leucocyte infiltrates and activated fibroblasts (Figure 5b). Control muscle from the same anatomical location was devoid of any visible pathological changes (Figure 5c).

3.2.2 | Melanized focal changes

Melanized focal changes were examined and classified into categories from 1 to 9 indicating the possible sequence at which they develop (Figure 6). A set of classification criteria was defined in order to differentiate different pathological features in the melanized changes in HE-stained sections. As melanized changes are polyphasic and different stages of inflammation might occur in the same section, the changes were categorized according to the dominating feature, in compliance with one of the histological categories. Thus, the macroscopic manifestations were histologically classified into the following categories: (a) no histological changes; (b) melano-macrophages in the

endomysium between apparently intact myocytes; (c) fibrosis in the endomysium without detection of melano-macrophages; (d) fibrosis in the endomysium with melano-macrophages; (e) melano-macrophages, fibrosis and presence of inflammatory cells in the endomysium; (f) (anticipated) old scar tissue with presence of inflammatory cells; (g) old scar tissue with presence of inflammatory cells and melano-macrophages; (h) focal granulomatous inflammation with presence of melano-macrophages; and (i) diffuse granulomatous inflammation with myocyte necrosis and myocyte regeneration and presence of melano-macrophages. The changes previously described by Larsen et al. (2012) would correspond to categories 8 and 9. The other categories have not previously been described.

3.2.3 | Macroscopic and histological results combined

When combining the macroscopic manifestations with the histological classification, diffuse granulomatous changes were only encountered in macroscopic manifestation grades 2 and 3 (Table 2). Further, in macroscopic manifestations graded 1, individuals with no observed histological changes were present (Table 2). However, grade 1 macroscopic changes displayed big variation in histological appearance, where all categories of histological changes were detected except diffuse granulomatous inflammation (Table 3). In macroscopic changes graded 2, diffuse granulomatous inflammation (category 9) could be detected; however, most of the category 9 changes were classified as grade 3 changes (Table 3).

3.2.4 | Other organs

No pathological changes occurred in the liver, spleen or head kidney at any of the samplings. Sections with pancreatic tissue, peritoneum and pyloric caeca revealed a moderate-to-severe peritonitis in the first two samplings (I and II) with a decreasing severity in the following samplings and a barely detectable level of severity in the last two samplings. This is in accordance with reactions towards intraabdominal vaccination (Mutoloki, 2004). The sideline including red and white muscle revealed scattered inflammatory foci in red muscle in some of the fish in Samplings I and II. Such changes occurred in 11 out of 31 fish in Sampling VII. The white muscle was generally unaffected. Sections of atrium and ventricle from Sampling I were without histological changes. In Sampling II, 3 out of 36 individuals had inflammatory infiltrates in the ventricular myocardium. In Sampling VII, similar changes occurred in 11 out of 31 individuals. The changes could not be associated with the presence of red and melanized changes. Both affected and apparently unaffected hearts occurred among fish with grade 3 changes.

TABLE 3 Number of melanized focal changes in each macroscopic grade (1–3) and microscopic category (1–9)

	1	2	3	4	5	6	7	8	9
Grade 1	9	10	1	6	6	1	3	1	0
Grade 2	0	7	0	6	8	1	0	3	7
Grade 3	0	0	0	1	2	1	0	0	5

3.2.5 | Special stains

Gram, Giemsa, PAS and Ziehl–Neelsen staining did not reveal any microorganisms in red or melanized changes.

3.2.6 | IHC and ISH

All granulomatous muscle changes were immunopositive for PRV, in concordance with earlier findings (Bjørgen, 2015), using an antibody targeting PRV protein (Figure 7A). Other, non-granulomatous, changes could appear both with and without PRV detection. ISH showed positively stained cells in the same locations and a similar distribution pattern as for IHC; however, detection of PRV σ1 proteins was generally more abundant than the detection of PRV RNA (Figure 7B).

3.3 | Experimental challenge study

The Ct values of PRV RNA in blood cells in the fish injected with PRV, that is, Group 1 injected with PRV-infected pelleted blood cells and Group 2 injected with purified PRV, were 22–24 at 3 wpc and approximately 30 at all later samplings. A few fish, mainly in Group 2 (injected with purified PRV), became PRV-negative at 12 wpc. In the cohabitant groups, that is, Groups 3 and 4, there was no detectable virus at 3 wpc, but from 6 wpc and thereafter, all fish in both groups had a Ct of approximately 20 in all samples, with a slight decreasing trend with time. All fish were positive in these groups after 3 wpc. The fish in the cohabitant groups, that is, those infected by natural route, had higher virus loads than the groups where the virus had been injected. Furthermore, there were no indications of clearing of the virus infection during the experiment for the cohabitant groups. In the groups of Tank 2, that is, Groups 5–9, where no infective material was administered, there was no detectable virus throughout the experiment.

No melanized focal changes occurred in any of the groups at any sampling after filleting; that is, melanized changes were not induced by experimental infection of PRV either in purified form or as in erythrocytes or as heat-inactivated PRV or heat-inactivated bacteria given i.m.

4 | DISCUSSION

This study describes the time-course development of melanized focal changes in a population of farmed Atlantic salmon in a regular commercial field setting during a PRV infection. Investigations on red and melanized focal changes have so far been conducted on material collected at slaughter; hence, the prevalence and severity of such changes during the production cycle in sea water have remained unknown. Here, we provide new information on

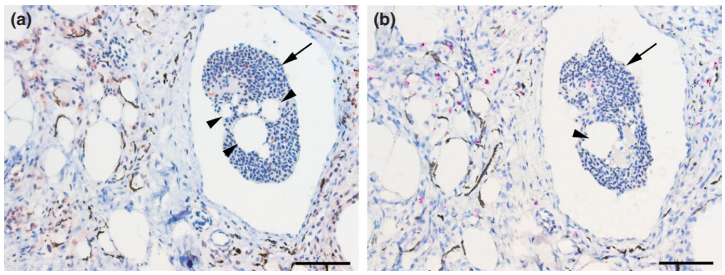

FIGURE 7 PRV presence in serial sections of a melanized focal change, category 9, with (a) IHC and (b) ISH. (a) PRV-positive cells (red) are present in the inflamed melanized tissue and in the blood (arrow). Note the adipocytes and vacuoles surrounded by elongated melanomacrophages. A negative imprint of lipid-resembling droplets is also evident within the blood in the vessel (arrowheads). (b) PRV-positive cells (pink) in the same locations as detected PRV by IHC. Staining is evident in the inflamed melanized tissue, but also in the peripheral blood (arrow). A negative imprint of lipid-resembling droplets is indicated (arrowhead). Scale bars = 50 μm [Colour figure can be viewed at wileyonlinelibrary.com]

the development of red and melanized changes from a large-scale field trial thought representative of most commercial fish farms in Norway. The presence of different features in melanized changes throughout the production cycle led to the establishment of a histological classification system. We also monitored the PRV status of the fish to further investigate the association between PRV infection and melanized focal changes described by Bjørgen et al., 2015. Our results strengthen the hypothesis for a transition from acute red to chronic melanized changes and further support the involvement of PRV in advanced melanized focal changes. *In situ* hybridization studies on PRV showed a great correlation with previous results (Bjørgen et al., 2015) and argue that PRV both persists and replicates in melanized focal changes, possibly explaining their chronic nature. Importantly, we also show that red focal changes and less severe melanized changes may occur without PRV presence.

RT-qPCR of blood samples became positive for PRV (2/61) in the fourth minor sampling (E4, 23 weeks post-sea transfer). Thereafter, the prevalence of PRV-positive blood samples increased steadily, and at the end of the observation period, all tested fish were PRV-positive. This indicated that PRV was exposed to the fish population after sea transfer and that it took at least 37 weeks from the initial PRV detection in gills (Sampling II) to widespread distribution and viraemia in the majority of the fish (Sampling VI). PRV belongs to the reoviruses; reo is an acronym for respiratory, enteric orphan, reflecting that these viruses were originally detected in the respiratory and enteric organs and that eventual causation of specific diseases was not evident. Enteric uptake of PRV has been indicated earlier (Hauge et al., 2016); however, these authors found no indication of oral entry as the fish were infected only through the intestine after anal intubation. Our finding that PRV was detected in gills before other sites is consistent with the gill as a port of entry for the virus. However, the Ct values from gills were high at the first positive samplings and one should be cautious to determine a port of entry based on that alone. As blood samples were negative in the same samplings, systemic infection with viraemia was probably not yet present. The density of fish farms was high in the area where the study farm is situated and rivers with wild migrating salmon are numerous, giving ample possibilities of spread of PRV through water or through contact with wild or escaped Atlantic salmon (Garseth, Fritsvold, Opheim, Skjerve, & Biering, 2013; Madhun et al., 2018).

The prevalence and the macroscopic and histological appearance of red focal changes remained remarkably stable throughout the observation period regardless of fish size, time post-vaccination, and ambient temperature or PRV infection status of the fish. The majority of the changes occurred cranio-ventrally in the fillet, that is, in the same region, and with similar gross appearance as melanized changes. The dominating anatomical localization combined with haemorrhages and myocyte necrosis is intriguing and could indicate local physiological dispositions for the placement. It was not possible to determine whether the changes started because of a haemorrhage following myocyte necrosis or vice versa, as a primary myocyte necrosis followed by haemorrhage. In some of the changes, only myocyte necrosis and haemorrhages occurred. In others, thought to be more advanced, varying amounts of leucocytes occurred. All of these histological forms were observed in all samplings. Histological and transcriptional examination for microorganisms in the changes gave no positive results with the exception of PRV, which appeared in muscle samples about 27 weeks post-sea transfer, while red focal changes occurred throughout the seawater phase. Consequently, there is no evidence that PRV infection induced the red changes.

The melanized focal changes were classified macro- and microscopically, and we provide a histological classification system for melanized changes. Investigations revealed a wide variety in the histological appearances within each macroscopic group. Importantly, the pathological features varied in concordance with the time the population had been in sea water. In the present sequential sampling, changes with the low macroscopic category dominated in the early samplings. Granulomatous inflammatory changes in macroscopic grade 3 occurred only in the last three samplings. The overall progression in severity of the spots with time spent in sea water has not previously been described. Due to the varying microscopic picture, all future studies addressing melanized focal changes should be supported by histological investigations.

Previous studies have reported that unvaccinated farmed fish develop melanized focal changes with the same prevalence at slaughter as vaccinated fish (Berg et al., 2012; Bjørgen et al.,2015; Larsen et al., 2014). However, in our current study, we observed the prevalence at different time points. In Sampling II, the overall prevalence of melanized focal changes more than doubled from Sampling I. The reason behind part of the increase might be attributed to vaccination and peritonitis (Mutoloki et al., 2004). Vaccine-induced side effects (moderate peritonitis) were detected in all fish from Samplings I and II and showed a decreasing severity in the following samplings. Peritoneal melanization is common in vaccine-induced peritonitis (Poppe & Breck 1997), and one could speculate that this temporary melanization of the peritoneal wall could be registered as a low-grade melanized change. This fits well with the fact that no severe melanized focal changes (grade 3) were observed at these samplings and that some muscle samples graded 1 showed no histological changes, perhaps indicating only melanization in the peritoneum. We thus believe that vaccination may account for the increased prevalence in Sampling II and that these changes may be of a temporary character without affecting the final prevalence at slaughter.

In some cases, we only encountered melano-macrophages that dispersed among seemingly unaffected myocytes. Their presence most likely explains the observed macroscopic discoloration. Other inflammatory changes were not observed. One may think that such manifestations may indicate the last phases of haematoma clearance. PRV was not detected in all red focal changes and, therefore, a plausible hypothesis is that in the absence of PRV, the healing process with invading macrophages will clear the changes. Conversely, PRV infection of leucocytes and possibly other cells at the site impairs the clearing process. The virus not only persists but also replicates in cells in the changes and the inflammatory process becomes chronic, as seen by the combined results of ISH and IHC. The lack of ability to eliminate the infection induces formation of granulomas attempting to seal off intruding antigen. This is supported by the ISH findings of replicating virus in all granulomatous changes examined. The melanin would protect the surroundings from the oxidative effect caused by the continuous inflammatory responses in the granulomas; that is, the more melanin and darker the spots, the more severe histopathological changes, which was supported by our findings. It would be ideal to compare these results with a population of fish remaining PRV-negative throughout the seawater period, but due to the widespread presence of PRV in Norwegian fish farms, such material remains unidentified. Though our results show that PRV is persistently present and replicates in macrophage-like cells within the chronic granulomatous changes, an alternative hypothesis would be that PRV in such changes is coincidental. The presence of PRV in granulomatous changes could be due to the widespread PRV-infected cells in the fish and not necessarily correlate with causation. This would imply another aetiology, and given the inflammatory response and granulomatous changes observed, this cause would most likely be of infectious origin. However, screening for viruses and bacteria has so far found that PRV is the only infectious agent consistently present in granulomatous melanized changes.

In the current study, 92% of all melanized changes were located in the cranio-ventral and mid-ventral parts of the fillet. It has to be speculated why the majority of changes occur at this site and whether the initial cause might originate in certain anatomical differences throughout the musculature. Interestingly, the percentage of fat content is considerably higher in these locations (47%) as compared to the more dorsal parts of the fillet (9%–18%) (Einen & Skrede, 1998). We did detect a negative imprint of extracellular lipid-like accumulations in both red and melanized focal changes and even in the lumen of blood vessels (Figure 7). The content of fat in the diet fed to farmed salmon has increased dramatically over the years, from about 15% in the 1980s to 35% today (Tacon, 2005). Concurrently, the amount of marine ingredients has dropped from around 90% in 1990 to 13% in 2013 (Ytrestøyl et al., 2015). The industry reports that the frequency of melanized changes has increased, partly documented in national surveys (Mørkøre et al., 2015). Another important factor reported to have increased over the same period is the occurrence of pancreas disease (PD) which is known to cause changes in white muscle (Mørkøre et al., 2011); however, melanized changes are present with approximately the same prevalence in the northern part of the country where PD has never been diagnosed. The increase in n-6 fatty acids from vegetable oils has caused concerns, and it is well known from humans that a high proportion of n-6 to n-3 fat in the diet shifts the physiological state in the tissues towards the pathogenesis of many diseases: prothrombotic, proinflammatory and proconstrictive (Simopoulos, 2008). In our study, the frequency of inflammatory changes in red and white muscle and myocardium increased with time. This tendency can possibly be associated with the ongoing PRV infection, but also with the growth of the fish and increased content of fat. An important observation from our previous investigation was that a PRV-positive population of fish farmed in in-house tanks was virtually free of both red and melanized changes (Groups F and G in Bjørgen et al., 2015). These fish were considerably smaller than the farmed fish in the other groups as they had a slower growth rate and this may have influenced the storage and distribution of fat in the fillet. Based on these observations, we speculate that the role of both fat in the diet and its distribution and content in fish should be given attention in future studies on melanized muscle changes.

We tried to establish an experimental in-house model for the induction of melanized focal changes by infecting fish with PRV. Artificial haemorrhages combined with PRV were induced by intramuscular injection of blood immediately after it was drawn from the same individual. The injection was in the dorsal muscles and not the cranio-ventral muscles and due to practical reasons; the duration of the experiment was 18 weeks, which is much shorter than the production period in sea water. A model for induction of melanized changes would be beneficial for many reasons, but the anticipated multifactorial nature of the development of the melanized changes, including anatomical placement, PRV infection, haemorrhages, myocyte necrosis, duration, feeding procedures and ingredients, should all be considered included (Lund et al., 2018).

The present study has shown that the occurrence of red focal changes in the seawater period seems stable and may turn into melanized focal changes, which accumulate over time. Red and melanized focal changes may be present without PRV being detected, but severe melanized forms were only found in association with PRV infection. The results support that the melanized focal changes in the abdominal region predominantly develop as a consequence of proceeding red focal changes. As these seemingly may occur without PRV, future research should reveal the cause for these changes, and subsequently prevent such changes from developing into chronicity. The prevention of PRV presence and replication seems to be crucial in the latter respect.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTION

HB planned the study, sampled material, carried out histological investigations (IHC and ISH) and wrote the manuscript; RH and AK sampled field material and commented on the manuscript; ØO planned the study and commented on the manuscript; DK carried out histological investigations (ISH) and commented on the manuscript; ER planned the experimental study, sampled experimental material and commented on the manuscript; and EOK supervised the study, sampled field material, analysed the results and edited the manuscript.

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Immunopathological characterization of red focal changes in Atlantic salmon (*Salmo salar*) white muscle

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ABSTRACT

Farmed Atlantic salmon (*Salmo salar*) are prone to various conditions affecting the quality of the fillet. A well-known but so far poorly understood condition is the focal red changes in muscle, often referred to as *haemorrhages*. Such changes are characterized by muscle necrosis, haemorrhages and acute inflammation. They can progress into *focal melanised changes*, a chronic inflammatory condition with melanin-producing leukocytes. The initial cause of intramuscular haemorrhages is unknown. In this study, we aimed to reveal some of their key immunological features. Samples of red focal changes were investigated by immunohistochemistry (IHC), *in situ* hybridization (ISH) and RT-qPCR for various immune markers. The results were compared with samples of melanised changes and control muscle, subjected to the same analyses. In all red changes, infiltrates with mononuclear cells were detected, consisting mostly of MHC class I/II$^+$ cells, but also of CD3$^+$ and CD8$^+$ cells. ISH studies on IgM showed few to moderate amounts of B-cells in red focal changes. Trends in the RT-qPCR showed upregulation of genes related to innate immunity in the red changes, whereas genes related to adaptive immunity were upregulated in the melanised changes. An important result was the significant downregulation of the anti-inflammatory cytokine IL10 in all red changes. Our findings indicate that we can rule out an auto invasive nature of the changes. The downregulation of IL10 at an early phase is a trait for the condition.

1. Introduction

Abundant melanin production may occur with chronic inflammatory conditions in ectothermic species. Chronic inflammation in the muscle of salmon (*Salmo salar*) may result in melanised focal changes in the fillet, which are commonly termed *black spots* (Larsen et al., 2012). With an occurrence of approximately 20 % of the fillets at slaughter, such changes are regarded as the most costly quality problem by the salmon industry (Mørkøre et al., 2015).

The hallmark of the inflammatory changes associated with melanised focal changes in skeletal muscle is the presence of melano-macrophages, which are melanin-containing macrophage-like cells found in ectothermic vertebrates (Bjørgen et al., 2019; Larsen et al., 2012). Most melanised focal changes occur in the cranio-abdominal region of the fillet and seem to develop from acute intramuscular haemorrhages, *i.e.* red focal changes, which over time progress into chronic melanised foci (Bjørgen et al., 2019, 2015). Red focal changes occur less frequent than melanised changes and have a prevalence of about 4 % throughout the production period in seawater (Bjørgen et al., 2019).

An earlier study of both red- and melanised focal changes collected at time of slaughter showed a range of macroscopic appearance from red to black discolouration, where all foci contained varying degrees of inflammatory cells, haemorrhage, myocyte regeneration, fibrosis and melanisation (Bjørgen et al., 2015). These findings linked the two conditions and strengthened the hypothesis of a transition from an acute red to a chronic melanised inflammatory manifestation. *Piscine orthoreovirus* (PRV) antigen was detected by immunohistochemistry (IHC) in all foci, whilst no other viruses were found, leaving PRV as the sole common viral denominator. PRV was detected in both acute haemorrhagic changes and in chronically inflamed changes with fibrosis and melanisation and was even found encapsulated within well-organised granulomas (Bjørgen et al., 2015). The virus was observed as

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Table 1
Primary antibodies used for immunohistochemical analysis.

	Antibody/dilution	Reactive against	Reference
PRV	Anti-PRV - σ1, 1:600	Capsid protein σ1	(Finstad et al., 2012)
	Anti-PRV - μNS, 1:2200	Non-structural protein μNS	(Haatveit et al., 2016)
Immune molecules	Sasa MHC class I F3-31, 1:20	Alpha 3 domain of MHC I alpha chain	(Hetland et al., 2010)
	Sasa MHC class II F1-12, 1:400	Beta 2 domain of MHC II beta chain	(Hetland et al., 2010)
	Anti-CD3, 1:400	Epsilon chain of the CD3 transmembrane protein	(Boardman et al., 2012)
	Sasa CD8 F1-29, 1:100	CD8α, a part of the CD8 complex	(Hetland et al., 2010)

globular cytoplasmic inclusions within macrophage-like cells and erythrocytes. These inclusions resemble viral factories which is the site of viral replication (Haatveit et al., 2016). A recent study on the development of discoloured muscle changes during the seawater phase has confirmed the presence of PRV in granulomatous melanised changes, but also showed that red- and low-grade melanised focal changes could occur without the presence of PRV, indicating that viral infection is not the initial cause of the myositis (Bjørgen et al., 2019). As studies on red focal changes are limited, it seems crucial to obtain more knowledge on these early-stage haemorrhages.

Red focal changes have been shown to occur in different macroscopic grades, ranging from small intramuscular haemorrhages to larger haematomas (Bjørgen et al., 2019, 2015). Histologically, all changes are characterised by similar pathological changes including acute necrosis of myocytes, extravascular erythrocytes, activated fibroblasts and endomysial and perimysial infiltration of leukocytes (Bjørgen et al., 2019). Based on their morphology, most leukocytes are thought to be macrophage-like cells undergoing myophagocytosis, but the presence of other leukocyte populations has so far not been studied.

With an unknown aetiology, this study aimed to reveal the immunopathological features of red focal changes. In humans, a number of different immune-related diseases are common in the musculoskeletal system (Dalakas, 2015), but such conditions have not been explored in fish. Here, we identify key innate, adaptive and anti-viral immune responses by applying immunohistochemistry (IHC), *in situ* hybridization (ISH) and RT-qPCR for different immune markers and genes. PRV is ubiquitous in farmed Atlantic salmon in Norway in the marine phase and the study was performed on PRV-positive fish. A group of fish with melanised focal changes (also PRV-positive) was included in the investigations, allowing a comparison between the acute and the chronic manifestation of the condition. As bacteria have previously been suggested to contribute to the pathogenesis of the condition (Krasnov et al., 2016), ISH was used to investigate for the presence of bacteria in red focal changes. Our study reveals important immunological features in red focal changes and provides novel information on the initial phase of this very costly condition.

2. Materials and methods

2.1. Fish groups and samples

Muscle samples from three different fish groups from our previous investigation were used in the current study (Groups A, C and H from (Bjørgen et al., 2015)). Group A consisted of samples of primarily melanised focal changes, while Group C consisted of samples of red focal changes. Both groups originated from fish that were positive for PRV-1 by RT-qPCR (Bjørgen et al., 2015). The groups came from different farm sites on the coast of Norway and were collected at fish abattoirs. A population of PRV-negative wild salmon without any detectable pathological changes (Group H) was acquired from the Drammen River in Eastern Norway. Details for the different groups have been published earlier (Bjørgen et al., 2015).

The groups were originally large, but for the purpose of in-depth immunological investigations, an arbitrary sub-selection from each group was made consisting of six red muscle changes from Group C (n = 6) and six melanised muscle changes from Group A (n = 6). These samples were used for both histological and gene expression studies. White muscle tissue from one fish in Group H (n = 1) served as negative control material for IHC/ISH experiments, as this sample was both PRV-negative and free from any observable pathological changes. For RT-qPCR experiments, muscle samples (6 from Group A and 6 from Group C with no visible changes) obtained from same anatomical location in the contralateral fillet were used as control material.

2.2. Histology and immunohistochemistry (IHC)

All samples were collected on 10 % phosphate-buffered formalin, routinely processed and paraffin-embedded after 24 h. Sections were cut 2 μm thick and mounted on glass slides, incubated for 24 h at 37 °C, de-waxed in xylene and rehydrated in graded alcohol baths and stained according to standard procedures with haematoxylin and eosin-staining (HE). The histological characteristics of the changes and controls have previously been described (Bjørgen et al., 2015).

Sections were cut 4 μm thick and mounted on glass slides (Superfrost + ©, Mentzel, Braunshweig, Germany), incubated for 24 h at 37 °C and thereafter for 30 min at 58 °C, de-waxed in xylene and rehydrated in graded alcohol baths before being transferred to distilled water. Sections were autoclaved in 0.01 M citrate buffer, pH 6.0 at 120 °C for 10 min, followed by treatment with phenylhydrazine (0.05 %; Sigma-Aldrich, St. Louis, MO, USA) for 40 min at 37 °C. The slides were rinsed three times in phosphate-buffered saline (PBS).

Nonspecific binding was prevented by blocking with goat normal serum diluted 1:50 in 5 % bovine serum albumin (BSA) in PBS. A primary antibody (Table 1) was diluted in tris-buffered saline (TBS) with 1 % BSA and added to the slides and incubated for 30 min. The sections were rinsed three times in TBS and incubated with a secondary antibody (EnVision© System kit; Dako, Glosrup, Denmark) for 30 min. The slides were washed three times in TBS and incubated with DAB or AEC for 7 or 14 min, respectively (EnVision© System kit), to evoke colour reaction (brown or red). Sections were washed with distilled water and counterstained with Mayer's haematoxylin for 1.5 min and mounted with Aquatex® (Merck KGaA, Darmstadt, Germany).

Negative control sections were incubated with 1% BSA or rabbit serum collected prior to immunization, instead of the primary antibody. For the PRV-staining, heart tissue collected from an heart and skeletal muscle inflammation (HSMI, where PRV is causative (Wessel et al., 2017)) challenge trial was used as positive control (Finstad et al., 2014).

2.3. Scoring of IHC

The muscle samples were scored using a semi-quantitative system. Each section was given a value between 0–3 according to the presence of immune-labelled cells. Value 0 was given when there was no observable labelling. When a low number of immune-labelled cells were present, the sample was scored with value 1. When labelling was moderate, with scattered single labelled cells in addition to focal or multifocal infiltrates, the sample was scored with value 2. When labelled cells were abundant in the section, with large numbers of single labelled cells in addition to multifocal infiltrates, the sampled was

scored with value 3.

2.4. ISH of IgM and 16S rRNA

Sections were cut 4 μm thick and mounted on glass slides (Superfrost + ©). To detect IgM RNAs, the slides underwent hybridization with a probe based on Atlantic salmon IgM sequence (Ssa-LOC106606767 targeting 219–1157 of XM_014203125) designed by ACDBio RNAscope® (Newark, CA). The sequence was chosen to detect RNA for both secreted and membrane-bound forms and all sub-variants of IgM. The probe targeting 16S rRNA was ordered from RNAscope® Catalog Probe List catalog number #464,461, confirmed as a universal target for bacteria. The method was performed using RNAscope RED 2.5 according to the manufacturer's instructions (Wang et al., 2012). Mid kidney was included as positive control tissue for the IgM probe. Head-kidney from fish with a systemic bacterial diagnosis (provided by the Norwegian Veterinary Institute) was used as control material for the 16S rRNA probe. RNAscope® Negative Control probe catalog number #701,021 was included to confirm absence of background and/or non-specific cross-reactivity in the samples.

2.5. RT-qPCR

Red and melanised focal changes (affected) and corresponding (unaffected) control muscle were collected and immersed into RNAlater solution. The samples were stored at −20 °C until RNA extraction.

The total RNA was extracted using QIAzol® Lysis Reagent. According to the manufacturer's recommendation, the extracted total RNA was dissolved in nuclease-free water. The total RNA was estimated using Nanodrop Spectrophotometer at the wavelength of 260 and 280 nm. Only RNA with an absorbance ratio A260:A280 greater than 1.9 were used for the reverse transcription. The total RNA was converted into cDNA as per the manufacturer's recommendations for QuantiTect® Reverse Transcription. Total RNA (750 ng) was aliquoted into 0.2 ml sterile PCR reaction tubes and DNA wipeout (2 μl) buffer was added. The total reaction volume was made up to 14 μl with nuclease free water. The cDNA reaction tube was incubated at 42 °C for 8 min in Thermal cycler and then immediately placed on ice for 10 min. The first-strand cDNA synthesis reverse-transcription cocktails (Quantiscript Reverse Transcriptase 1 μl Quantiscript RT buffer 5 × 4 μl and RT primer mix 1 μl) were aliquoted 6 μl into each reaction tube. The conditions used were 37 °C for 30 min and 95 °C for 3 min. After successive completion of reverse-transcription reactions, the cDNA cocktail was stored on ice before performing qPCR analysis and reverse-transcription reactions cocktail stored at −20 °C for long-term storage. The cDNA was used as a template for further amplification using RT-qPCR with specific primers targeting key innate, adaptive and anti-viral immune genes (Table 2).

Evaluation of gene expression was carried out using specific gene primers in Agilent Mx3005P-Stratagene qPCR instruments. cDNA of each experimental group was used in triplicate along with EF1AB as an internal control. A negative control reaction was performed for all the primers excluding of the cDNA template. The amplifications were carried out in a 96 well plate with a total volume of 13 μl in each well, using cDNA, 2 pmol of each gene-specific primer pair with TaqMan™ Universal PCR Master Mix (Thermo Fisher Scientific) and DEPC water. The following thermal cycling conditions were used: 95 °C for 10 min, 95 °C for 15 s, 58 °C for 15 s and 60 °C for 60 s for 40 cycles. After amplification, relative expression levels of CD4-2a, CD8, MHC class I, MHC class II, Viperin, Mx1, IFNγ, TNFα, IL1b, IL10 and Casp3 were estimated. The relative fold of induction was determined by ΔΔCT method, allowing a quantification of the expression of the genes of interest against the reference gene (EF1AB) by means of fold change. The fold (mean ± SD, n = 6) change was calculated using the formula 2- ΔΔCT. The testing method used was One-way ANOVA. Statistical significance was calculated by the Dunnett's multiple comparisons test.

3. Results

3.1. Immunohistochemistry – red focal changes

Two different PRV proteins were targeted by IHC: the structural protein σ1 and the non-structural protein μNS. The latter is not a part of viral particles and thus indicates active viral replication. In red changes, the σ1 labelling scored between 2–3, *i.e.* moderate to high amounts of immune-labelled cells (Table 3), while μNS labelling revealed a similar pattern (Fig. 1A). The μNS^+ cells displayed a distinct, cytoplasmic and globular label (Fig. 1B).

Moderate to high amounts (scored 2–3) of MHC class I and II^+ cells were detected in all changes (Table 2). MHC class I^+ cells were widely distributed in and around necrotic myocytes in the connective tissue (Fig. 2A), phenotypically resembling macrophage-like cells, with a single oval nucleus located eccentrically. Focal accumulations of MHC class I^+ were frequent (Fig. 2B). All myocytes and erythrocytes were MHC class I^-, but the endothelial lining of intra-muscular capillaries were MHC class I^+, consistent with results from previous studies (Hetland et al., 2011, 2010). Scattered MHC class II^+ cells were found throughout the changes both in the myomeres and in the connective tissue of the myosepta (Fig. 2C). MHC II^+ cells were also encountered in focal accumulations (Fig. 2D) where MHC class I reactivity was seen in the same foci.

Immunolabelling for T cells varied significantly within the red focal changes, though immunolabelled cells were detected in all individuals (Table 3). Large amounts of $CD3^+$ cells (scored 3) were detected in three out of six samples. $CD8^+$ cells occurred in different amounts in four of the samples. Only one of the changes had large amounts of $CD8^+$ cells (scored 3). Serial sections of a highly reactive red focal change showed severe myocyte necrosis and multifocal inflammation (Fig. 3A). The infiltrate contained numerous cells labelled for PRV (Fig. 3B) and displayed strong labelling for MHC class II (Fig. 3C). T cells appeared abundantly in the same infiltrate (Fig. 3D) and many cells were $CD8^+$ (Fig. 3E). The unaffected virus negative control muscle was negative for all antibodies, except MHC class I, where occasional immunolabelled cells were present in the perimysium (data not shown).

3.2. Immunohistochemistry – melanised focal changes

Immunolabelling for PRV with two different antibodies (anti-σ1 and anti-μNS) displayed immunolabelled cells in all melanised focal changes (data not shown). The score varied (from 1 to 3) with the stage of the inflammation: severe muscle changes with degeneration, necrosis and multiple leukocyte infiltrates generally received a high score, while in highly fibrotic changes with less degeneration and fewer leukocytes had only scattered immunolabelled cells and a lower score. Nodular granulomas were found in several changes (Fig. 4A). μNS^+ cells were detected among inflammatory cell infiltrates but were also present centrally in the granulomas (Fig. 4B), indicating replication of virus within such changes. Further, the granulomas displayed phenotypic features typical of an active immunological site, with strong immunolabelling for MHC class I (Fig. 4C), a central core of MHC class II^+ cells (Fig. 4D) with surrounding $CD3^+$ cells (Fig. 4E) and occasional $CD8^+$ cells (Fig. 4F).

3.3. In situ hybridization – IgM in red and melanised focal changes

IgM^+ cells were found in all changes in varying amounts. Most of the labelled cells had a characteristic lymphocyte-like phenotype with a prominent round nucleus and scant cytoplasm. In the red changes, few to moderate numbers of labelled cells were seen in most changes except one which contained many IgM^+ cells (Fig. 5A). In all melanised changes, many IgM^+ cells were detected within the granulomatous inflammation (Fig. 5B). Mid kidney control tissue contained numerous IgM^+ cells. Negative controls showed no labelling of cells.

Table 2
Sequences of primers and probes used in quantitative real-time RT-PCR analysis.

Gene	Sequence (5´- 3´)	Reference
$EF1A_B$	F-TGCCCCTCCAGGATGTCTAC R-CACGGCCCACAGGTACTG P-FAM-AAATCGGCGGTATTGG-MGB	(Olsvik et al., 2005)
CD4-2a	F-GCCCCTGAAGTCCAACGAC R-AGGCTTCTCTCACTGCGTCC P-FAM-CCGCACACTAGAGGGTCCACCACG-BHQ	(Aas et al., 2014)
CD8a	F-ACTTGCTGGGCCAGCC R-CACGACTTGGCAGTTGTAGA P-CGACAACAACAACCACCACG-BHQ	(Aas et al., 2014)
MHC-I	F-GGAAGAGCACTCTGATGAGGACAG R-CACCATGACTCCACTGGGGTAG P-TCAGTGTCTCTGCTCCAGAAGACCCCCT-BHQ	(Aas et al., 2014)
MHC-II	F-CCACCTGGAGTACACACCCAG R-TTCCTCTCAGCCTCAGGCAG P-FAM-TCCTGCATGGTGGAGCACATCAGC-BHQ	(Aas et al., 2014)
IL-1b	F-GCTACCACAAAGTGCATTTG R-GAGGTTGGATCCCTTTATGC P-FAM-CCATTGAGACTAAAGCCAGACCTGTAG-BHQ	(Moldal et al., 2014)
IL-10	F-GAAACATCTTCCACGAGCTG R-GTCCAGCTCTCCCATTGC P-FAM-TTCTCCTGTAAGAAACCGTTTGACATC-BHQ	(Aas et al., 2017)
Mx1	F-GATGCTGCACCTCAAGTCCTATTA Rev-CACCAGGTAGCGGATCACCAT P-FAM-GCTGATCAGATTCCCATGGT-BHQ	(Wessel et al., 2017)
Viperin	F-AGCAATGGCAGCATGATCAG R-TGGTTGGTGTCCTCGTCAAAG FAMAGTGGTTCCAAACGTATGGCGAATACCTGBHQ	(Austbø et al., 2014)
TNFa	F-GCAGCTTTATGTGCGGCAG R-TTTTGCACCAATGAGTATCTCCAG P-FAM-TGGAAGACTGGCAACGATGCAGGA-BHQ	(Moldal et al., 2014)
IFNg	F AGGACACACCTGCAGAACCTG R AGTTTGGAGGCTTTCTCGTAGATG FAM-AGTCCAGGGGAAGGCTCTGTCCG-BHQ	(Austbø et al., 2014)
Casp3[†]	F-AGTGAAGGTTGCCAATGACC R-ACATTGCCATCTGTGCCATA	NM_001139921.1

[†] SYBR green primers that were analysed using corresponding SYBR green EF1AB housing keeping primers.

Table 3
Scoring of IHC from muscle samples. Values 0-3 indicate respectively no labelled cells (0); few labelled cells (1); moderate amounts of labelled cells (2); and large amounts of labelled cells (3).

	Fish	PRV σ1	PRV μNS	CD3	CD8	MHC class I	MHC class II
	Control	0	0	0	0	1	0
Red focal changes	I	2	3	3	2	3	2
	II	3	3	3	1	1	3
	III	2	2	2	0	2	2
	IV	2	1	1	0	2	3
	V	3	3	3	3	3	3
	VI	3	2	1	1	2	2
Melanised focal changes	I	3	3	3	2	2	3
	II	1	1	3	1	2	2
	III	2	1	1	1	2	2
	IV	3	2	2	1	2	3
	V	3	3	2	1	2	2
	VI	2	2	2	1	2	3

3.4. Investigations on bacterial presence - in situ hybridization targeting 16S rRNA and Gram stain

Scattered bacteria-like structures were occasionally detected within intact myocytes and in cells in the endomysium in some red (Fig. 6A) and melanised changes. These bacteria-like structures were not commonly found in relation to pathological changes but appeared randomly distributed throughout the tissue in both red and melanised changes. Most changes did not show labelling for 16S rRNA (Fig. 6B). Gram stain in parallel sections did not reveal the presence of bacteria. Positive controls demonstrated labelling for bacteria (Fig. 6C).

3.5. RT- qPCR analysis of red and melanised focal changes

Gene expression of selected immune- and anti-viral genes were investigated in both red and melanised changes and in unaffected control muscle. The individual variation was high for most genes, but with some trends for different groups (Fig. 7). For genes related to acute, innate immune responses, IL1b and TNFα expression were upregulated in some individuals in both red and melanised changes and were highest in the red changes. There was a highly significant down-regulation of IL10 in the red changes ($P < 0.0001$), while a modest increase of IL10 was present in the melanised changes ($P < 0.05$).

For genes related to adaptive immunity, a statistically significant increase in CD4 was detected in both red and melanised changes when compared to unaffected muscle. CD8, MHC class I and II were generally upregulated in the melanised changes (statistically significant for MHC class II, $P < 0.05$) and a similar trend was not found in the red changes.

For innate antiviral genes, Mx1 and Viperin expression varied a lot between individuals and no clear trends were detected in either group. INFγ was downregulated in all melanised changes ($P > 0.05$), while the red changes showed a diverse INFγ expression. An increase in gene expression of Casp3 was seen in all red changes, though not statistically significant.

4. Discussion

The immune response in melanised focal changes in Atlantic salmon has been investigated previously in both histologic and transcriptomic studies (Krasnov et al., 2016; Larsen et al., 2012). However, this is the first report on the immune characterization of red focal changes. In comparison with melanised focal changes, the immunological features

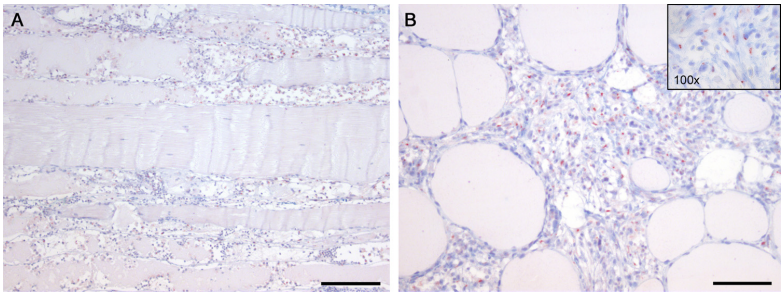

Fig. 1. PRV μNS immunolabelling of a red focal change. A) Abundant amounts of PRV$^+$ cells (red) in the necrotic muscle tissue, in addition haemorrhage and degenerated myocytes. Scale bar = 100 μm. B) PRV$^+$ cells (red) are present in a dense infiltrate of leukocytes. High magnification image shows distinct and globular immunolabelling in the cytoplasm of mostly macrophage-like cells. Scale bar = 50 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

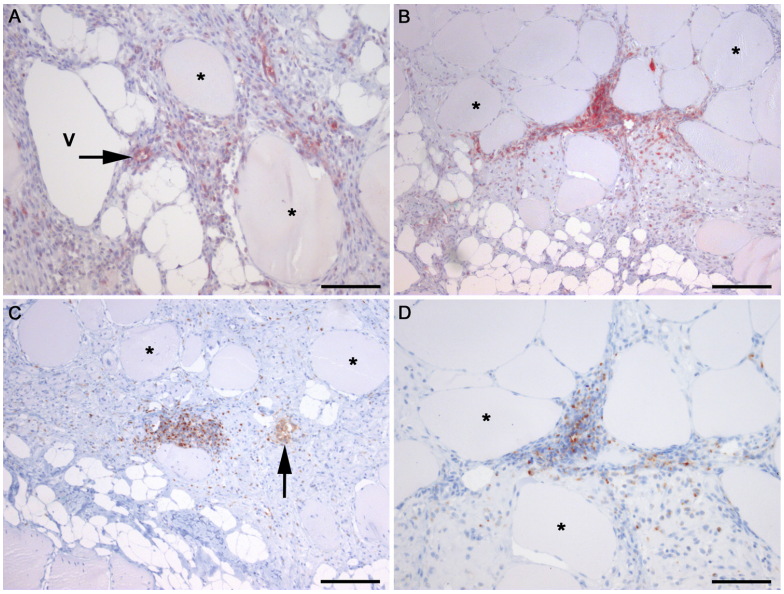

Fig. 2. MHC class I (A and B) and II (C and D) immunolabelling in red focal changes. A) Abundant amounts of MHC class I$^+$ cells (red) are present in the inflamed muscle tissue. The cells are distributed in a scattered manner, as well as in focal clusters. Immunolabelling is also seen in the endothelial lining of intramuscular vessels (arrow). V = vacuole. Scale bar = 50 μm. B) A dense, focal cellular infiltrate (red) is present in the necrotic muscle tissue. Degenerated myocytes with MHC class I$^+$ cells (red) are evident near the fatty tissue of the myosepta. Scale bar = 100 μm. C) Scattered and focal accumulations of MHC class II$^+$ cells (brown) appear abundantly in the necrotic muscle tissue. Immunolabelled cells in a degenerated myocyte are indicated (arrow). Scale bar = 100 μm. D) A focal infiltrate of MHC class II$^+$ cells. Scale bar =50 μm. Examples of myocytes are indicated by asterisks (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

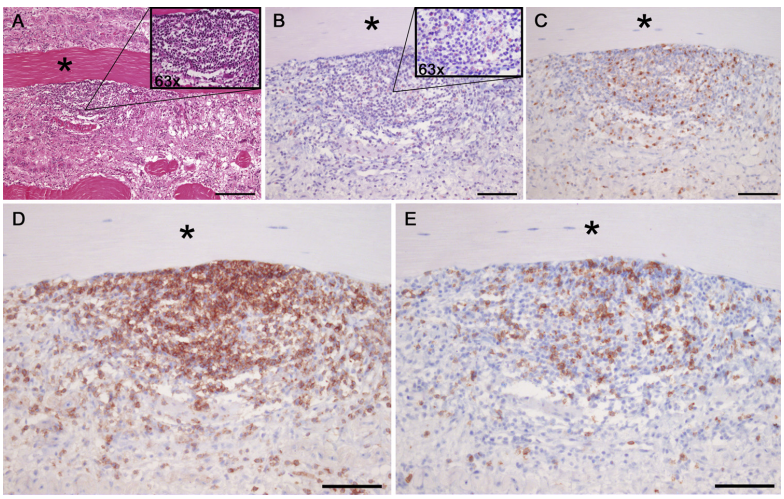

Fig. 3. Serial transverse sections of a red focal change. A large, central infiltrate of leukocytes is present and the change scored 3 on PRV-, MHC class I and II-, CD3- and CD8 IHC labelling. Asterisk in A-E marks a myocyte. A) A degenerated and necrotic musculature with a dense focus of leukocytes beneath an intact myocyte (asterisk). Higher magnification (63 x) image in the upper right corner. H&E stain. Bar = 100 μm. B) Multiple PRV$^+$ macrophage-like cells (red) in an area with abundant leukocytes. PRV σ1 immunolabelling. Bar = 50 μm. C) Multiple MHC class II$^+$ cells (brown) in an area with abundant leukocytes. MHC class II immunolabelling. Bar = 50 μm. D) Large amounts of CD3$^+$ cells (brown) within an infiltrate of leukocytes. CD3 immunolabelling. Bar = 50 μm. E) Multiple CD8$^+$ cells (brown) in an infiltrate of leukocytes. CD8 immunolabelling. Bar = 50 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

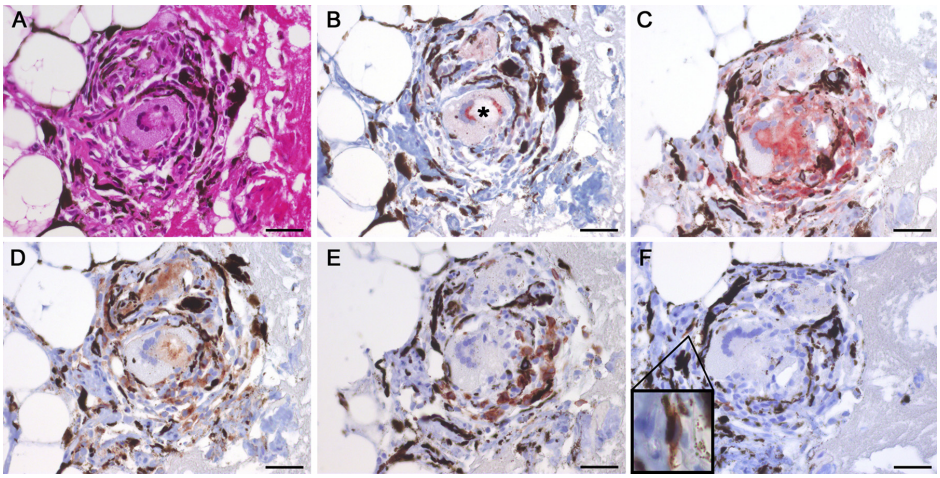

Fig. 4. Immunolabelling of a nodular granuloma in a melanised focal change. A) A round to oval granuloma located in the periphery of a myotome towards the myoseptum. The cell-rich granuloma is heavily melanised and contains a central core with necrotic debris and a multi-nucleated giant-cell. H&E staining. B) PRV$^+$ cells (red) are present in the granuloma centre (asterisk). Immunolabelled cells are evident in relation to a multi-nucleated giant cell. PRV μNS immunolabelling. C) Abundant MHC class I$^+$ cells (red) in both peripheral and central parts of the granuloma. MHC class I immunolabelling. D) Numerous MHC class II$^+$ cells are present within the granuloma (brown). MHC class II immunolabelling. E) Immunolabelled T lymphocytes surround the central core the granuloma and are associated with the fibrous capsule of the granuloma. CD3 immunolabelling. F) A few CD8+ cells are present in the in the outer fibrous capsule of the granuloma. High magnification picture of an immunolabelled cell is shown in the lower left corner. CD8 immunolabelling. Scale bar (A-F) = 20 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

of red focal changes suggested a tissue site in transition from an acute to chronic inflammation. IHC showed a varied presence of immune cells in red changes while melanised changes were dominated by granulomatous inflammation, including highly organized granulomas. The non-structural PRV protein μNS was detected in structures resembling viral factories, which are specialized structures where PRV replicates and new viral particles are assembled. The viral factory-like structures were found in both red and melanised changes. Furthermore, immune genes associated with innate, adaptive and innate anti-viral immunity showed that expression of the anti-inflammatory cytokine IL10 was down regulated (statistically significant $P < 0.0001$) in all red changes and upregulated in all melanised changes.

The samples in our study were collected at slaughter and thus PRV-positive, and representative for farmed Atlantic salmon in Norway, where PRV is ubiquitous (Løvoll et al., 2012). The non-structural protein μNS was targeted by IHC to address the presence of replicating PRV. The protein was found in both red and melanised changes in various amounts indicating an on-going replication even in the highly organized granulomas. Positive signal occurred as cytoplasmic inclusions, *i.e.* resembling viral factories (Haatveit et al., 2016). The presence of PRV specific RNA has been shown previously by ISH in granulomatous melanised changes (Bjørgen et al., 2019); here we indicate that PRV replication occurs in red changes. Importantly, we know that red changes can develop without PRV infection and therefore, we cannot attribute the presence of replicating virus as the cause for the condition and that there may be several causes for development of red changes (Bjørgen et al., 2019). On the other hand, the development of chronic melanised changes has been associated with local persistence and replication of PRV (Bjørgen et al., 2019), thus possibly acting as the constant trigger of inflammation.

The presence of immune cells was investigated in both red and melanised changes. Various amounts of MHC class I and II and T cells were detected in all changes and they often occurred in highly reactive foci. This was reflected by the PCR results, where substantial individual variation was observed, however, the lack of homogeneity in these changes is an intrinsic unknown factor for the interpretation of the RT-qPCR results. Diversity within the changes could easily be visualised by histology, however, such information is lost in tissue extractions and PCR analysis. The appearance of focal accumulations of immune cells, as seen in the immunolabelling, could have a large effect on the RT-qPCR results pending upon the sampling. Therefore, histological investigations are necessary when studying heterogenic tissue like

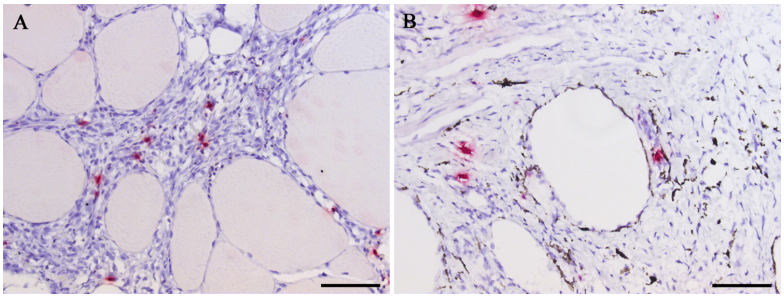

Fig. 5. *In situ* hybridization – IgM in red and melanised focal changes. A) IgM$^+$ cells in a highly reactive inflammatory focus in a red focal change. Bar =50 μm. B) IgM$^+$ cells scattered around a vacuole surrounded by numerous melano-macrophages. Bar = 50 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

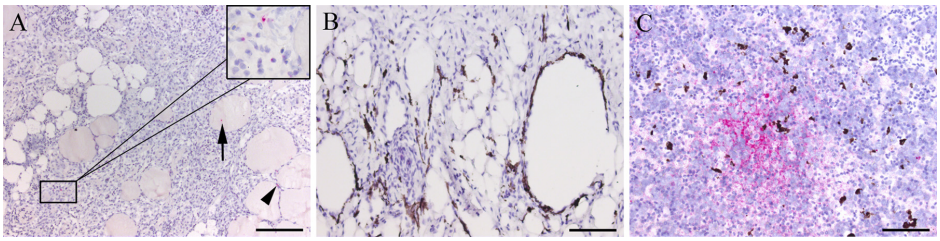

Fig. 6. *In situ* hybridization - 16 s rRNA in red and melanised focal changes. A. Scattered 16S rRNA-labelled structures in a red focal change, appearing within intact myocytes (arrow) and in cells in the endomysium (arrowhead). Enlarged area shows some labelled structures within the inflammatory changes. Bar = 100 μm. B. A chronic and heavily melanised change shows no labelling for 16S rRNA. Bar = 50 μm. C. Control head-kidney from fish with systemic bacterial disease, showing a severe, focal accumulation of 16S rRNA-labelled structures. Bar = 50 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

melanised changes, where the information achieved by transcriptional analysis may be confusing.

MHC class I (and class II) is normally undetectable in mammalian myocytes under physiological conditions. However, MHC class I is up-regulated in certain pathological conditions, especially in inflammatory muscle diseases (Nagaraju, 2001). Studies on mammals have shown that muscle cells can act as facultative antigen-presenting cells and should be considered as active participants, rather than passive targets, of immune reactions (Nagaraju, 2001; Wiendl et al., 2005). CD8-positive T cells, *i.e.* cytotoxic T cells, and activated macrophages invade the muscle tissue and it is assumed that the auto invasive cytotoxic T cells recognise muscle-derived self-peptide antigens presented in MHC class I on the myocyte surface, causing necrosis (Wiendl et al., 2005). We hypothesized that this could be the case also with the onset of the inflammatory condition in salmon muscle, but we never detected MHC class I positive myocytes in the focal red changes where myocyte necrosis was abundant. We therefore consider it unlikely that MHC class I-driven myositis is the initial process in the pathogenesis of red focal changes.

Previous investigations using different staining procedures have

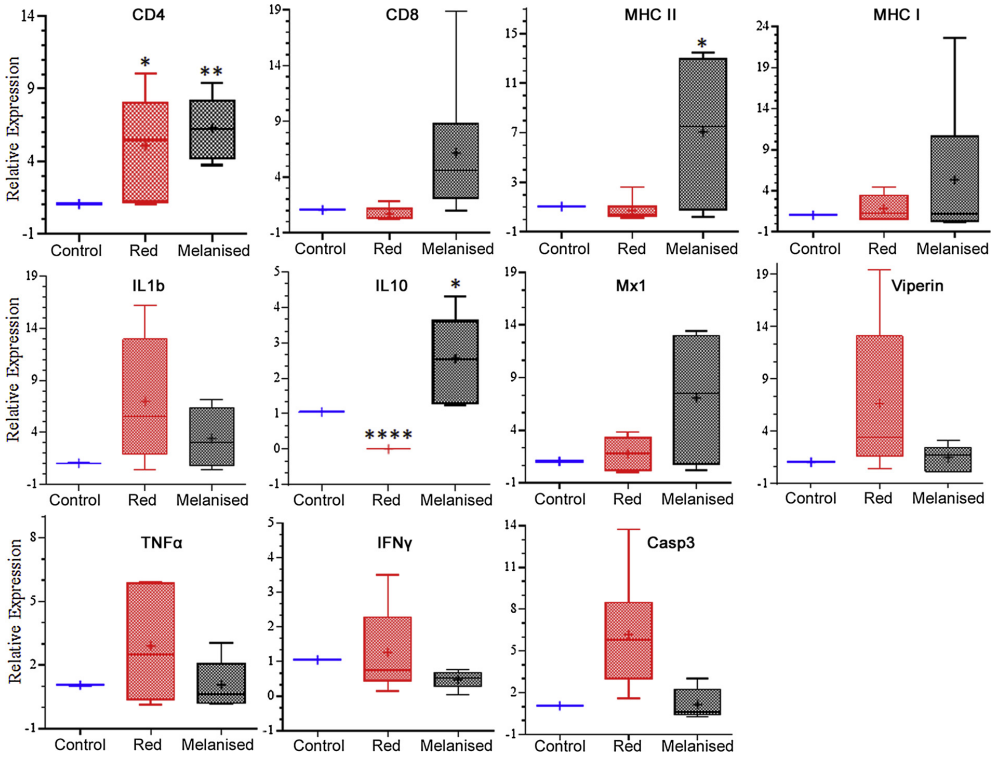

Fig. 7. RT-qPCR of selected genes in control muscle and in red and melanised focal changes. Bar graphs showing relative fold change of mRNA expression of CD4-2a, CD8, MHC class II, MHC class I, IL1b, IL10, Mx1,Viperin, TNFα, IFNγ and Casp3 in focal red (red bars) and melanised (dark grey bars) changes and control muscle (blue bars) from the same individuals normalized against EF1AB. The asterisks represent a significant difference from the control (****$p < 0.0001$, **$p < 0.01$ & *$p < 0.05$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

never revealed bacteria in the changes (Bjørgen et al., 2019, 2015; Koppang et al., 2005; Larsen et al., 2012). However, bacteria have been suggested by others to be a component in the pathogenesis of focal muscle changes (Krasnov et al., 2016). Therefore, the sensitive technique of ISH (RNAscope©, ACD Bio) targeting 16S rRNA, a universally conserved target for detection of bacterial RNA, was performed in parallel with Gram stained sections to detect the possible presence of bacteria. In red and melanised changes, scattered bacteria-like 16S rRNA structures were occasionally seen within seemingly un-affected myocytes and in cells in the endomysium; however, we were not able to verify these results with Gram staining. The appearance and distribution of 16S rRNA-positive signals could very well be bacteria-like structures; however, the positive signals were not seen in relation to the pathological changes. The presence of 16S rRNA-positive structures in the periphery of the tissue sections were interpreted as bacterial contamination following sampling. This, in addition to the generally low presence or total absence of detection in most samples, indicates that bacteria are not involved in the pathogenesis of focal muscle changes. Replicating virus, however, was constantly found within granulomatous, melanised changes, and was a feature consistent with the chronic nature of the condition which is in accordance with our previous findings (Bjørgen et al., 2019). The μNS PRV staining was observed mainly in macrophage-like cells, indicating that the virus is able to replicate in the scavenger cells in an inflammatory focus. The increase in Viperin and Mx expression in red and black changes, respectively, indicate a dominating anti-viral response. In addition, the expression of Caspase3, which plays an essential role in apoptosis, increased in acute red changes. This might be an attempt to prevent viral infection and replication, as apoptosis is a common host defense strategy in several viral infections (Orzalli and Kagan, 2017).

The progression from acute red to chronic melanised changes was demonstrated by the shift in the inflammatory response. The innate immune genes investigated included several pro- and anti-inflammatory interleukins (IL1β, TNFα, IFNγ and IL10) chosen based on their role in inflammatory and anti-inflammatory reactions in fish (Secombes et al., 2011). Genes encoding for adaptive immune molecules included markers for both non-professional and professional antigen-presenting cells (MHC class I and II) and genes for T cell surface proteins (CD4 and CD8). Trends in the RT-qPCR results showed that innate immune parameters were generally more highly expressed in the acute red changes, while expression of adaptive immune parameters was generally higher in the chronic melanised changes. The strong and statistically significant downregulation of IL10 in the red changes was a major finding in these investigations. Our results reveal the importance of this immune parameter as a key regulator of the immune responses in this condition. As the function of IL10 is downregulation of immune responses, its absence of expression in the acute changes indicates a swift onset of defense mechanisms as a response to the observed injury. Concurrent trends were seen in the expression of several pro-inflammatory cytokines (TNFα, IFNγ and IL1) which were generally higher expressed in red focal changes, though not statistically significant. Among previous studies reporting on IL10 in teleost fish, Collet et al. (Collet et al., 2015) investigated the expression of IL10 in Atlantic salmon peripheral blood following experimental infection with infectious salmon anemia virus (ISAV) showing induction at a late stage of the infection. Similarly, Ingerslev et al. (Ingerslev et al., 2009) found a significant upregulation of IL10 24 days post an intraperitoneal challenge with infectious pancreatic necrosis virus (IPNV). Both of these studies seem to correspond well with our results, where IL10 was downregulated in all red changes followed by an upregulation in all melanised changes. Importantly, both above-mentioned studies were controlled experiments with fish in the same phase of the infection. In our case, the samples were not obtained in a controlled experimental environment, meaning that the fish was selected only on the basis of presence of different muscle changes, and their infection history was unknown. Therefore, we cannot exclude the effects of other pathogens or external factors, despite all fish being PRV infected. However, we know from a previous study that red changes can occur without virus infection and that the impact of PRV appears to be more important in severely melanised changes (Bjørgen et al., 2019). Nevertheless, our results are highly consistent, pointing to a significant role of IL10 in the condition.

In conclusion, the red focal changes are dominated by haemorrhage and myocyte necrosis. A prominent downregulation of IL10 was seen in all red changes. PRV was present and shown to replicate in the changes. The impact of the virus is uncertain, and this study cannot conclude with respect to causes for the onset of the condition. Nevertheless, the possibility of MHC class I-driven myositis seems unlikely as IHC targeting MHC class I was negative in myocytes in all red focal changes. The immunopathological characterization of red focal changes supports the notion that the red focal changes are an acute form that develops into a chronic inflammation where granulomas with replicating PRV are a consistent feature.

Authors' contributions

HB: Planning, sampling of material, histological experiments and analysis and writing of manuscript; KS: RT-qPCR analysis and commented on the manuscript; GG: Histological analysis and commented on the manuscript. CMP: Planning and commented on the manuscript. ER: Planning and commented on the manuscript; EOK: Planning, sampling of material, commenting on the manuscript and co-ordinating the overall work.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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IV

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PRV-1 Infected Macrophages in Melanized Focal Changes in White Muscle of Atlantic Salmon (*Salmo salar*) Correlates With a Pro-Inflammatory Environment

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Melanized focal changes in white skeletal muscle of farmed Atlantic salmon, "black spots", is a quality problem affecting on average 20% of slaughtered fish. The spots appear initially as "red spots" characterized by hemorrhages and acute inflammation and progress into black spots characterized by chronic inflammation and abundant pigmented cells. *Piscine orthoreovirus* 1 (PRV-1) was previously found to be associated with macrophages and melano-macrophages in red and black spots. Here we have addressed the inflammatory microenvironment of red and black spots by studying the polarization status of the macrophages and cell mediated immune responses in spots, in both PRV-1 infected and non-infected fish. Samples that had been collected at regular intervals through the seawater production phase in a commercial farm were analyzed by multiplex fluorescent *in situ* hybridization (FISH) and RT-qPCR methods. Detection of abundant inducible nitric oxide synthase (iNOS2) expressing M1-polarized macrophages in red spots demonstrated a pro-inflammatory microenvironment. There was an almost perfect co-localization with the iNOS2 expression and PRV-1 infection. Black spots, on the other side, had few iNOS2 expressing cells, but a relatively high number of arginase-2 expressing anti-inflammatory M2-polarized macrophages containing melanin. The numerous M2-polarized melano-macrophages in black spots indicate an ongoing healing phase. Co-localization of PRV-1 and cells expressing $CD8^+$ and MHC-I suggests a targeted immune response taking place in the spots. Altogether, this study indicates that PRV-1 induces a pro-inflammatory environment that is important for the pathogenesis of the spots. We do not have indication that infection of PRV-1 is the initial causative agent of this condition.

Keywords: Atlantic salmon, black spots, macrophage polarization, *Piscine orthoreovirus*, red spots

INTRODUCTION

Melanized focal changes in the white skeletal muscle of farmed Atlantic salmon (*Salmo salar*), "black spots", has emerged as a phenomenon that is found on average in 20% of the Atlantic salmon slaughtered at Norwegian processing plants (1). Fish affected with spots appear clinically healthy, and the condition is therefore regarded as a quality problem rather than associated with a disease state. Most melanized changes locate to the cranio-ventral and mid-ventral parts of the fillet, which could indicate an anatomical and physiological disposition for the condition (2). However, the etiological cause of the focal melanization remains unknown.

The black spots are primarily observed at slaughter of seawater farmed Atlantic salmon (3), and there are no reports that such spots are common in wild fish. Histologically, black spots appear heterogenous. The more severe black spots are classically characterized as chronic inflammatory reactions of granulomatous character, where macrophages are the dominating cell type, and the presence of melano-macrophages gives the black discoloration (3). In a longitudinal study where the presence of spots was followed through the seawater production phase in a commercial farmed salmon population, it was concluded that red spots preceded the formation of black spots (2). The term red spots refer to foci in the white muscle assumed to be intramuscular hemorrhages. The red spots were found to have a stable low prevalence in the production period, while the black spots accumulated over time in the fish population in seawater (2). Histopathological classification of the melanized spots show that they develop over the time the fish population has spent in sea water, and the most serious granulomatous inflammatory changes appear a few months before slaughter and are associated with *Piscine orthoreovirus* 1 (PRV-1) (2). Aggregation of macrophages and other immune cells forming granulomatous structures in the black spots indicate a long-term activation of the immune response (4).

Both immunohistochemistry and *in situ* hybridization methods have demonstrated presence of PRV-1 in melanized foci (2, 4). PRV-1 is a very common infection in farmed Atlantic salmon in the marine phase (5). The presence of PRV-1 in the black spots has been associated with the severity of the spots (2, 4). However, due to the increasing prevalence of PRV-1 infection in farmed Atlantic salmon with time spent in seawater, an alternative hypothesis would be that the presence of melanized changes is coincidental and not caused by PRV-1 infection. In line with this, some macroscopic dark spots are found in fish without detectable PRV-1 infection, but histologically these spots do not show the same chronic inflammatory and granulomatous reactions (2). In black spots with histopathological changes, classified as granulomatous changes, PRV-1 seems to be a consistent finding (2).

PRV virions are naked particles of 70 nm-large icosahedral capsids encompassing the genome of ten double stranded (ds) RNA segments, categorized into long (L1-L3), medium (M1-M3) and small (S1-S4) segments. There are three recognized subtypes of PRV. PRV-1 is mainly found in Atlantic salmon where it may cause heart and skeletal muscle inflammation (HSMI) (6). Following infection of PRV-1 in Atlantic salmon, the virus replicates to high titers in its main target cell, the erythrocyte (7, 8), and subsequently high virulent variants of PRV-1 infect cardiomyocytes leading to the cardiac inflammation observed during heart and skeletal muscle inflammation (HSMI) (9).

Previous studies have indicated that Atlantic salmon does not clear the PRV-1 infection, and the acute infection develops into a persistent, low productive infection (10). In the persistent phase, PRV-1 infection can be found in circulating erythrocytes and renal erythroid progenitor cells, but also in macrophages and melano-macrophages in kidney and spleen (11). In the melanized spots and in the granulomatous reactions of the more severe black spots in particular, PRV-1 is found in macrophage-like cells, melano-macrophages and erythrocytes (2, 12). This could indicate that the infected cells have a role in the pathogenesis of the melanized changes. Melano-macrophages primarily reside in the spleen and head kidney of teleost fish, where they can cluster to form so-called melano-macrophage centers, but they may also migrate to inflamed tissues (13).

Macrophages are often classified according to their polarization rather than their tissue location. The M1 type macrophages are classically activated and polarized by IFN-γ signaling. They produce a pro-inflammatory microenvironment by secreting inflammatory cytokines, and have the capacity to inactivate intracellular pathogens through, among other factors, the action of nitric oxide (NO) and reactive oxygen species (ROS) (14, 15). Presence of M1 macrophages in an area with infection suggests that macrophage polarization have occurred through sensing of danger signals (16, 17). M1 macrophages are a common phenotype of phagocytes during a cell mediated immune response (18).

The M2 macrophages, on the other hand, are anti-inflammatory and are central in wound healing and tissue repair (19, 20). M2 macrophages can be activated by anti-inflammatory cytokines (IL-4 or IL-13) (21) and their main functions are to generate extracellular matrix and polyamines for cell growth and division, in addition to protein synthesis necessary for the healing process (22). There are many indications that the polarized macrophage phenotypes exist also in teleost fish (23, 24), and the presence of inducible nitric oxide synthase (iNOS2) and arginase-2 (Arg2) have been defined as M1 and M2 specific markers, respectively (22).

Interaction between cytotoxic T-lymphocytes (CTLs) and the antigen presenting complex MHC-I on the target cell surface can initiate the killing of target cells by the actions of granzymes and perforins produced by CTLs (25, 26). Involvement of CTLs in the host defense mechanism against PRV-1 infected cells is indicated in HSMI (27) and spots development (12). The specific co-localization pattern of PRV-1 and the targeted response of these immune cells can be exposed through multiplex *in situ* hybridization method.

This study aimed to characterize the polarization of macrophages in red and black spots by multiplex fluorescent *in situ* hybridization (FISH) method and to study the correlation of markers of macrophage polarization, MHC-I and CD8 expressing cells with PRV-1 infection. The reduction of the relative number of PRV-1 infected cells through the spots' stages indicated an elimination of PRV-1 infected cells in the melanized focal spots

in Atlantic salmon. Transformation of red spots into black spots is associated with the emergence of melano-macrophages of M2 phenotype in the white skeletal muscle.

MATERIAL AND METHODS

Samples From Field Trial

Atlantic salmon smolts with an average weight of 110 g were transferred to sea in a commercial setting at Svåsand, Hardanger, Norway, as earlier described (2). The fish were sampled regularly throughout the seawater period and screened visually for presence of red and black spots in the white muscle (2). Formalin fixed samples of red and black spots had been categorized and graded based on macroscopic appearance and the PRV-1 infection status of the population had been monitored by RT-qPCR of gill, spleen and muscle samples by PatoGen Analyse, Ålesund, Norway as earlier described (2). The population was PRV negative upon transfer to sea and the first PRV positive fish were detected at 23 weeks post transfer. At 48 weeks post transfer about 98% of the sampled fish were PRV-1 positive. The samples used in the present study were collected at 4 and 52 weeks post sea transfer, i.e. prior to PRV-1 infection and after the population was near completely infected with PRV-1, in this context referred to as PRV negative and positive, respectively. The seawater temperature was 11-11.5°C at samplings.

The samples were collected from white muscle of the cranio-ventral part of the fillet and were no spots (normal tissue), macroscopic red spots and black spots (**Table 1**). Macroscopically the spots were graded 1-3 where grade 1 was very faint discoloration, 2 was a distinct but not severe discoloration and 3 was a prominent and severe discoloration (2).

RNA Extraction and RT-qPCR

Total RNA was extracted from a 25 mg sample of the tissues from all fish from each group as shown in **Table 1** using 0.65 ml QIAzol Lysis reagent (Qiagen, Hilden, Germany). Tissues were homogenized using 5 mm steel beads in a TissueLyzer II (Qiagen) for 2 x 5 min at 25 Hz. Chloroform was added and the aqueous phase collected for automatic RNA isolation using a RNeasy Mini QIAcube Kit (Qiagen), eluting RNA in 50 µl RNase free water. RNA concentrations were determined in a Nanodrop ND-100 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Thereafter, RNA was stored at -80°C until further use.

cDNA was synthesized from 1 µg total RNA by using Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's guidelines. In short, the procedure included elimination of genomic DNA and incubation at 42°C for 30 min with RT mastermix including reverse transcriptase enzyme and RNAse inhibitor. Quantitative PCR was performed in duplicates in 96-well plates, using a reaction volume of 12 µl with 15 ng cDNA input per well, and the Maxima SYBR Green/ROX qPCR Master Mix-2x (Thermo Fisher Scientific). Thermal conditions were set for an initial denaturation at 10 min/95°C and 40 cycles of amplification at 15 sec/95°C, 30 sec/60°C and 30 sec/72°C. Melting curve analysis were included to ensure assay specificity. Elongation factor (EF1ab) was used as reference gene (28). No-template control (NTC) were run on the same plate as negative control. The cut off value was set to Ct 35, and fold induction of genes of interest was determined against the reference gene and control samples (29). Primers (**Table 2**) were designed using Vector NTI Advance™ 11 software (Thermo Fisher Scientific), and AlignX application (Vector NTI Advance™ 11 Package, Invitrogen Dynal AS) was used for sequence alignments. (**Table 2**)

TABLE 1 | Samples selected from red and black spots.

Category	PRV status	Grading	Sampling time (weeks after transfer to sea)
Black spot	Positive (n = 6)	Grade 1-3 black spots	52
	Negative (n = 6)	Grade 1-2 black spots	4
Red spot	Positive (n = 5)	Grade 1-3 red spots	52
	Negative (n = 6)	Grade 1-3 red spots	4
No spot	Positive (n = 6)	No macroscopic lesion	52
	Negative (n = 4)	No macroscopic lesion	4

Statistical Analysis

Fold change (2- ΔΔCt formula) medians for genes of interest were compared in all groups, using non-parametric Mann-Whitney test due to small sample number, to display differences among the groups. GraphPad Prism version 9.0 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis and graphical layouts. $p \leq 0.05$ was considered as significantly different.

Histology

Samples for histological examination were selected from PRV-1 infected and uninfected fish populations with or without macroscopic red and black spots (**Table 1**). Selection criteria for the uninfected population with red and black spots was spot grade level 2 (n = 1) because no uninfected fish had grade 3 level black spots. Samples from PRV-1 infected fish with red and black spots had grade 3 (n = 2). Samples from uninfected fish without macroscopic lesion were selected randomly and used as negative control, whereas samples from infected fish without spot were selected based on highest PRV-1 level (**Table S1**). Formalin fixed paraffin embedded (FFPE) tissue section (2 µm thickness) was dehydrated by gradual ethanol baths followed by xylene washing for paraffin clearance. Rehydration of the sections were performed for subsequent staining with hematoxylin and eosin (H&E staining). Standard procedures were followed (32). Bright field microscopy (Carl Zeiss Light Microscopy System with Axio Imager 2 - Carl Zeiss AG, Oberkochen, Germany) was performed for imaging.

Fluorescent *In Situ* Hybridization (FISH)

Sample Pretreatment

FFPE sections were sliced with 5 µm thickness from tissue samples and mounted on Superfrost plus (Thermo Fisher Scientific) slides. Slides were baked at 60°C for 2 h in a HybEZ™ II oven (Advanced Cell Diagnostics, catalog #321720)

TABLE 2 | List of specific primers for genes of interest.

Genes	Primer	Conc.	Sequence (5'-3')	Amplicon (bp)	Accession No.
iNOS2*	F	400 nM	CATCGGCAGGATTCAGTGGTCCAAT	135	XM_014214975.1
	R		GGTAATCGCAGACCTTAGGTTTCCTC		
Arg2*	F	400 nM	CCTGAAGGACTTGGGTGTCCAGTA	109	XM_014190234.1
	R		CCGCTGCTTCCTTGACAAGAGGT		
MHC Class I (30)	F	400 nM	CTGCATTGAGTGGCTGAAGA	175	AF504022
	R		GGTGATCTTGTCCGTCTTTC		
CD8α (31)	F	400 nM	CACTGAGAGAGACGGAAGACG	174	AY693393
	R		TTCAAAAACCTGCCATAAAGC		
Granzyme A (31)	F	400 nM	GACATCATGCTGCTGAAGTTG	81	BT048013
	R		TGCCACAGGGACAGGTAACG		
EF1αb (28)	F	500 nM	TGCCCCTCCAGGATGTCTAC	57	BG933897
	R		CACGGCCCACAGGTACTG		

**Amplification efficiency (E) of newly designed primers were calculated for iNOS2 (E = 0.98) and Arg2 (E = 1.02).*

followed by deparaffinization with 100% ethanol and fresh xylene baths. Samples were pretreated with hydrogen peroxide for 10 min at RT, boiled with RNAscope antigen retrieval reagent (Advanced Cell Diagnostics, catalog #322000) for 15 min at 99°C, and then incubated with RNAscope protease plus reagent for 15 min at 40°C in the HybEZ™ II oven. Hydrophobic barrier was made around the tissue section using Immedge hydrophobic barrier pen (Vector Laboratories, Burlingame, CA).

Multiplex *In Situ* Probe Hybridization

RNAscope® Multiplex fluorescent V2 assay kit (Advanced Cell Diagnostics catalog #323100) was used for simultaneous detection of up to three different RNA targets. Probes (**Table 3**) were designed against; PRV-1 L3 segment (Advanced Cell Diagnostics catalog #537451) iNOS2 (Advanced Cell Diagnostics catalog #548391); Arg2 (Advanced Cell Diagnostics catalog #548381) CD8α (Advanced Cell Diagnostics catalog #836821); Granzyme A (Advanced Cell Diagnostics catalog #836841) and MHC-I (Advanced Cell Diagnostics catalog #836831). Probes against Peptidylpropyl Isomerase B (PPIB) (Advanced Cell Diagnostics, catalog #494421) was used as reference gene for RNA integrity of the target samples. Dihydrodipicolinate reductase (DapB), a bacterial gene from *Bacillus subtilis* (Advanced Cell Diagnostics catalog #310043) was used as negative control gene to assess cross-reactivity and background noise. Probes were mixed and hybridized for 2 hrs at 40°C in the HybEZ™ II oven. Amplification steps (Amp1-Amp3) were performed according to the manufacturer's protocol. Opal fluorophores (**Table 2**) (Akoya Biosciences, CA, United States) were prepared and diluted (1:1500) using tyramide signal amplification (TSA) buffer (Advanced Cell Diagnostics catalog #322809) provided in the kit. Each probe was assigned a fluorophore, having a different emission and excitation range to distinguish each output signal (**Table 3**). Also, every probe was developed, labeled, and blocked separately by incubating with RNAscope® multiplex Fluorescent Detection Reagents v2 (catalog #323110) and diluted Opal fluorophores in a sequential order as per manufacturer recommendations. Each section was counter stained by adding DAPI (fluorescent DNA stain) for 30 sec at room temperature. Mounting was performed by adding 1-2 drops of Prolong Gold antifade mounting reagent (Thermo Fisher Scientific). Imaging was performed in a TCS SP8 gSTED confocal microscope (Leica microsystems GmbH, Mannheim, Germany).

RESULTS

Histology of Red and Black Focal Changes

In samples from white muscle from non-infected fish without visible spots, unaltered and intact myocytes were seen (**Figure 1A**). In samples of non-spot tissue from PRV-1 infected fish, mild myocyte degeneration was observed to some extent along with presence of infiltrating leukocytes (**Figure 1B**).

In red spots the white skeletal muscle tissue showed moderate to severe bleedings, and mild degeneration to moderate myocyte necrosis was observed in uninfected and infected fish, respectively (**Figures 1C, D**). The red spots from PRV-1

TABLE 3 | List of probes and corresponding fluorophores used in FISH.

	Probe	Target Region (bp)	Fluorophores	Emission/Excitation Wavelength (nm)	Channel*
Target	PRV-L3	415–1379	Opal 520 (FP1487001KT)	494/525	C1
	iNOS2	2–949	Opal 620 (FP1495001KT)	588/616	C2
	Arg2	1332–2053	Opal 690 (FP1497001KT)	676/694	C3
	CD8α	8-1033	Opal 620 (FP1495001KT)	588/616	C2
	GzmA	3-1088	Opal 690 (FP1497001KT)	676/694	C3
	MHC-I	2-2321	Opal 620 (FP1495001KT)	588/616	C2
Control	PPIB	20–934	Opal 520 (FP1487001KT)	494/525	C1
	DapB	414–862	Opal 520 (FP1487001KT)	494/525	C1

**Channels signify the specific labeling of each fluorophore separately for their excitation and emission properties.*
Accession numbers; PRV-L3- KY429945; PPIB- NM_001140870; DapB- EF191515, for the other genes the acc. nos. are listed in ***Table 2****.*

infected fish differed from the noninfected fish by infiltration of leukocytes and scattered appearance of adipocytes. In black spots, melanin was found in both groups (**Figures 1E, F**), however, the presence of macrophage-like cells with histologically observable melanin content, referred to as a melano-macrophages, in the samples of the infected fish were more prominent and widespread. Moreover, granulomatous changes in the black spots were observed in PRV-1 positive fish (**Figure 1F**) and never in the uninfected fish.

In Situ Localization of Differentiated, Polarized Macrophages and PRV-1 in Focal Changes

a. Uninfected fish

The positive and negative controls, i.e. using the PPIB and DapB probes, are shown in **Figures S1**, **S2**, respectively. Tissues with macroscopic appearance of red focal changes from uninfected fish showed no iNOS2 specific staining, but some Arg2 positive

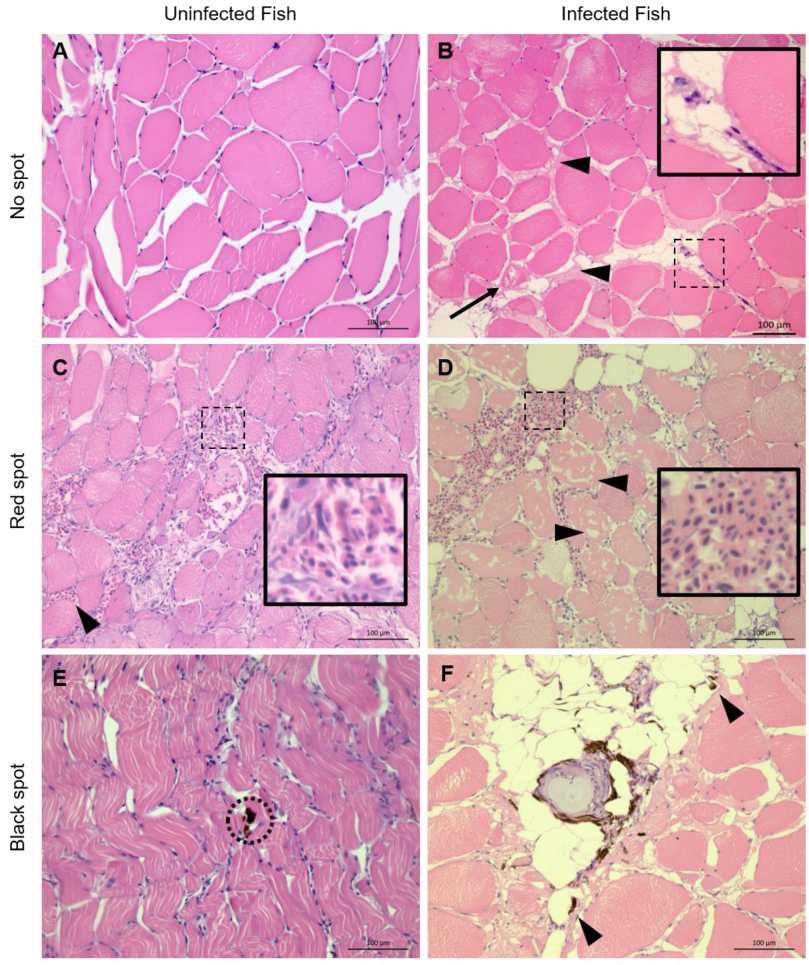

FIGURE 1 | Representative histological sections from white skeletal muscle of noninfected and PRV-1 infected Atlantic salmon HE stained. **(A)** Myocytes with no observable changes. **(B)** Myocytes with mild degeneration (arrow) and presence of inter-myocytial fluid (arrowheads) and few infiltrating leukocytes (inset). **(C)** Uninfected fish, red focal change shows areas with moderate (inset) and minor (arrowhead) hemorrhages. Note minor degeneration in some myocytes with infiltrating macrophages. **(D)** Red focal change in PRV-1 infected fish with large hemorrhage (inset) and massive myocyte necrosis (arrowheads). **(E)** Black spot in uninfected fish. Sporadic occurrence of melano-macrophages (black) in seemingly otherwise non-affected tissue (dotted circle). **(F)** Black spot in infected fish with a typical granulomatous change surrounded with melano-macrophages (black). Arrowheads point to scattered melano-macrophages. Scale bar = 100 µm.

cells (**Figure S3**). Similarly, sections with the macroscopic appearance of black spots from uninfected fish showed low number of iNOS2 or Arg2 positive cells (**Figure S4**). No staining was seen in areas without spots from uninfected fish (**Figure S5**). No PRV-1 signal was detected from noninfected fish groups having spots or no spots (**Figures S3–S5**).

b. Red spot. Early phase. PRV-1 infected.

In red focal changes there were hemorrhages (**Figure 2A**) containing a large number of nucleated cells (**Figure 2B**). Hemorrhages analyzed by FISH showed PRV-1 in a few erythrocytes (**Figure 2C**). Numerous iNOS2 positive macrophages, i.e. M1 type polarized macrophages, were surrounding the hemorrhage (**Figure 2D**), but co-localization of PRV-1 and iNOS2 were not seen (**Figure 2E**). There was no staining for Arg2, i.e. M2 type polarized macrophages (**Figure 2F**). Due to the low presence of M1 activated macrophages and lack of organization of the hemorrhages, this appearance was assessed as being an early phase of the red spots.

c. Red spot. Intermediate phase. PRV-1 infected.

In red focal changes from infected fish, where the changes were assessed as more advanced and infiltrating cells were seen between the myocytes (**Figure 3A**). The large number of extravasal erythrocytes of the early phase was not present (**Figure 3B**). Co-localization of PRV-1 and iNOS2 was observed among infiltrating cells found between myocytes (**Figures 3C, D**). There was no staining with Arg2 (**Figure 3F**). Due to the high presence of M1 activated macrophages and the organized appearance of the hemorrhages, but lack of melano-macrophages, this was assessed as being an intermediate phase of the red spots.

d. Red spot. Late phase (transition between red and black spots). PRV-1 infected.

In another region from the same red spot sample, as displayed in **Figure 3**, there were some scattered deposits of melanin (**Figure 4A**). In these areas, there was a modest number of PRV-1 positive cells (**Figure 4B**.) Here, Arg2 specific transcripts were detected (**Figure 4C**), with co-localized PRV-1 staining (**Figure 4D**). Detection of Arg2 was only observed in melano-macrophages found in the sporadic melanin deposits (**Figure 4E**). The commencement of M2 type melano-macrophage detection was assessed as an indication of transition from red to black spots.

e. Black spots. PRV-1 infected

In macroscopic black spots, larger deposits of melanin were seen (**Figure 5A**), and there was a moderate density of cells (**Figure 5B**). PRV-1 stained cells were mainly seen in the area of melanization (**Figures 5C, D**). Scattered areas of iNOS2 positive cell populations were detected in the melanized focal changes (**Figure 5E**), but these only partly co-localized with PRV-1 staining (**Figure 5F**). Arg2 positive cells were also seen in the PRV-1 infected area together with melanin presence (**Figure 5G**), showing some co-localization of PRV-1 and Arg2 (**Figure 5H**). A number of melano-macrophages with PRV-1 were detected. Arg2 positive transcripts were primarily detected in melano-macrophages, but were also present in non-melanized M2 macrophages (**Figure 5I**).

f. No focal changes, PRV-1 infected

In PRV-1 infected fish, samples from areas in white muscle without spots showed co-localization of iNOS2 and PRV-1 (**Figures 6A–D**), while Arg2 and PRV-1 only partly overlapped (**Figures 6E, F**). The staining of iNOS2 and Arg2 did not overlap.

Presence of CD8$^+$ and MHC-1 Positive Cells

a. Red Focal Changes, PRV-1 infected

In situ labeling revealed a mild influx of CD8$^+$ cytotoxic T lymphocytes (CTLs) in the bleeding area of red focal changes in PRV-1 infected fish (**Figure 7A**). Some of the CD8+ cells were also positive for PRV-1 staining, whereas

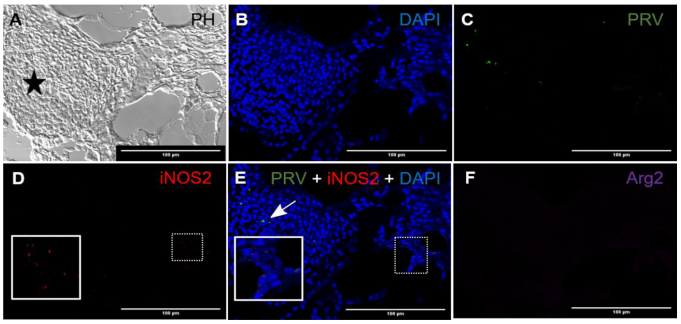

FIGURE 2 | *Fluorescent in situ hybridization of PRV-1, iNOS2 and Arg2 in red focal changes (early phase).* **(A)** Phase contrast image showing a large hemorrhage (star). **(B)** DNA staining of the cells by DAPI (blue). **(C)** Presence of a few PRV-1 (green) positive cells in the hemorrhage. **(D)** iNOS2 (red) specific transcripts detected in a limited number of cells surrounding a peripheral blood vessel. **(E)** Merged image showing presence of PRV-1 (arrow) but no co-localizing in the M1 macrophage (inset). **(F)** Arg2 (purple) specific transcripts (M2 type macrophages) were undetected. Scale bar = 100µm.

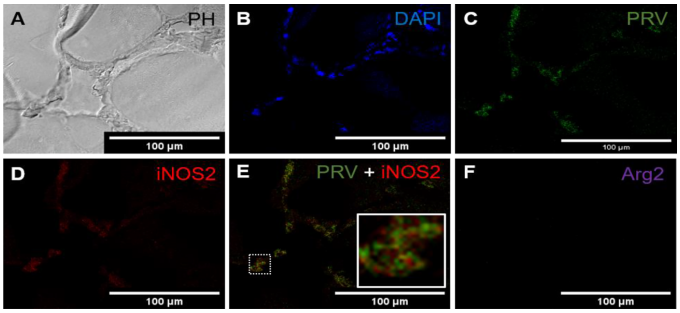

FIGURE 3 | *Fluorescent in situ hybridization of PRV-1, iNOS2 and Arg2 in red focal changes (intermediate phase).* **(A)** Phase contrast image showing infiltrating cells between myocytes. **(B)** Nuclei DNA staining of the cells with DAPI (blue). **(C)** Presence of PRV-1 (green) in infiltrating cells in between myocytes. **(D)** Presence of iNOS2 (red) in infiltrating cells between myocytes. **(E)** Merged image showing co-localization (inset) of PRV-1 and iNOS2 (yellow). **(F)** Arg2 transcripts (purple) were not detected. Scale bar = 100µm.

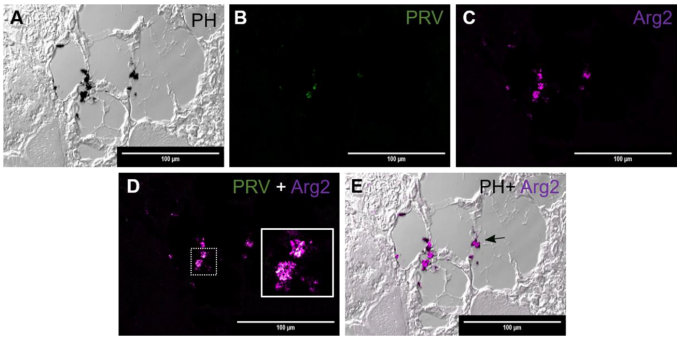

FIGURE 4 | *Fluorescent in situ hybridization of PRV-1 and Arg2 in red focal changes (late phase).* **(A)** Phase contrast image showing structure of the analyzed area and melanin deposit. **(B)** Sporadic presence of PRV-1 (green) in melanized area. **(C)** Arg2 (purple) positive cells **(D)** Merged image showing PRV-1 and Arg2 co-localization (white in the inset). **(E)** Merged image showing Arg2 positive staining of melano-macrophages (arrow). Scale bar = 100µm.

other CD8-positive cells were present around infected cells (**Figures 7A, B**). Granzyme A transcripts were detected in both $CD8^+$ (**Figures 7A–C**) and other cell populations in the infected area (**Figures 7A, B**). Numerous MHC-I positive cells were present at the bleeding site (**Figures 8A–C**), with a limited number also being PRV-1 infected. PRV-1 did not appear to co-localize with MHC-I (**Figures 8B, C**).

b. Black Focal Changes, PRV-1 infected

CD8+ cells were detected in the areas with melanin deposits. Some PRV-1 infected cells were also detected in this area, but the staining did not co-localize (**Figure 9**). Granzyme A specific transcripts were co-localized with CD8+ (**Figures 9C, D**) but also found in other cell subsets (**Figure 9B**). Numerous MHC-I positive cells were detected around a vacuolar area surrounded by melano-macrophages and some PRV-1 infected cells, showing high melanin deposits (**Figures 10A, B**). PRV-1 did co-localize with some MHC-I stained cells (**Figure 10C**).

Gene Expression in Red and Black Spots

a. iNOS2 and Arg2

iNOS2 expression was low in PRV positive (median Ct 30.7), non-spot samples (2 folds)while it was significantly increased (approximately 10 folds, MWU value = 0, n1 = 4, n2 = 5, p-value = 0.0079) in PRV positive (median 27) red focal changes (**Figures 11A**, **S6**). In contrast, iNOS2 was not upregulated in the samples from red focal changes of uninfected fish. In the black focal changes, iNOS2 expression was at the same level as in non-spot samples. Arg2 expression was significantly upregulated in all of the target groups, especially in PRV-1

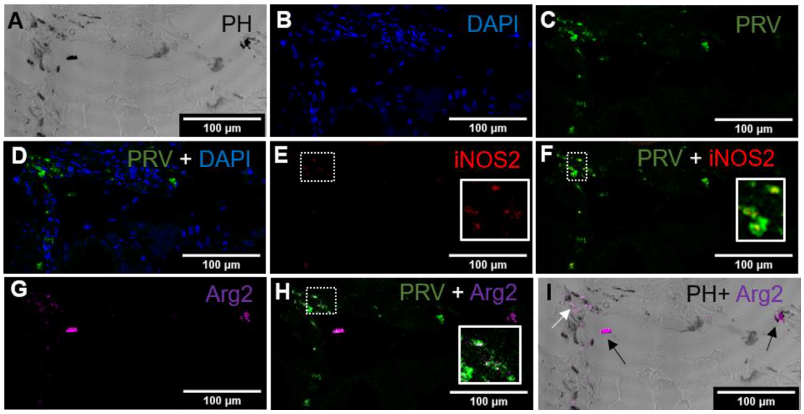

FIGURE 5 | *Fluorescent in situ hybridization of PRV-1, iNOS2 and Arg2 in black focal changes (late phase).* **(A)** Phase contrast image presence of melanin in the infected area. **(B)** Nuclei DNA stained with DAPI (blue). **(C)** PRV-1 (green) detected in severe melanized area **(D)** Merged PRV-1 and DAPI. Number of PRV-1 positive cells compared to total number of cells were low. **(E)** Few iNOS2 (red) positive cells detected at the infected area **(F)** Merged image showing co-localization (yellow in inset) of PRV-1 and iNOS2. **(G)** Presence of Arg2 (purple) positive cells. **(H)** Co-localization of PRV-1 and Arg2 positive cells (white in inset). **(I)** Localization of Arg2 specific transcripts in melanized M2 melano-macrophages (black arrows) and non-melanized M2 macrophages (white arrow). Scale bar = 100µm.

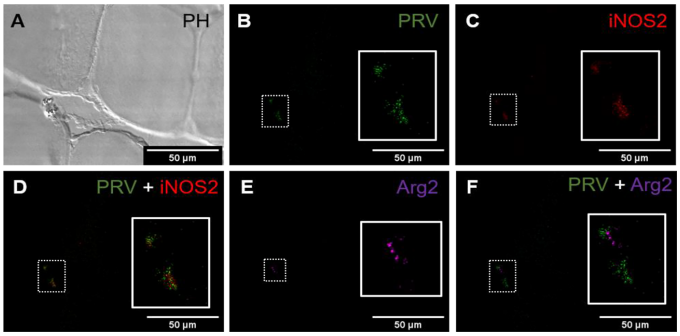

FIGURE 6 | *Fluorescent in situ hybridization of PRV-1, iNOS2 and Arg2 in areas without spots, from PRV-1 infected fish.* **(A)** Phase contrast image showing cells structure. **(B)** PRV-1 (green) specific transcripts detected between muscle cells. **(C)** iNOS2 (red) positive cells in the same area as PRV-1. **(D)** Merged image showing co-localization of PRV-1 and iNOS2 (yellow, insert). **(E)** Arg2 (purple) positive cells were detected partly in same area as PRV-1. **(F)** Merged image of PRV-1 and Arg2 show Arg2 positive cells surrounding PRV-1 infected cells (inset). Scale bar = 50µm.

infected groups. Arg2 was upregulated both in infected, no spot samples (6.5 folds, MWU value = 0, n1 = 4, n2 = 6, p-value = 0.0048) and in red focal changes without PRV infection (4.7 folds, MWU value = 1, n1 = 4, n2 = 6, p-value = 0.0095), compared to the uninfected, no-spot control. Both iNOS2 and Arg2 expression were at the highest level in the PRV infected fish with red focal changes. But in black focal changes only Arg2 expression, in contrast to iNOS2, was significantly upregulated (MWU value = 0, n1 = 4, n2 = 6, p-value = 0.0048) in PRV-1 infected group (median 27.7) (**Figures 11A**, **S6**).

b. CD8α, GzmA and MHC-I

There was a trend towards upregulation of the CD8α gene in the red and black focal changes (**Figure 11B**) but this was not statistically significant. Granzyme A expression level was significantly upregulated in PRV-infected groups with red (16.5 folds, MWU value = 0, n1 = 4, n2 = 5, p-value = 0.0079) and black focal changes (approx. 15 folds, MWU value = 2, n1 = 4, n2 = 5, p-value = 0.0489). Non-infected groups showed no significant induction of CD8α and granzyme A. Increased expression of MHC-1 was spotted in all fish groups infected with PRV-1, compared to non-infected groups, and

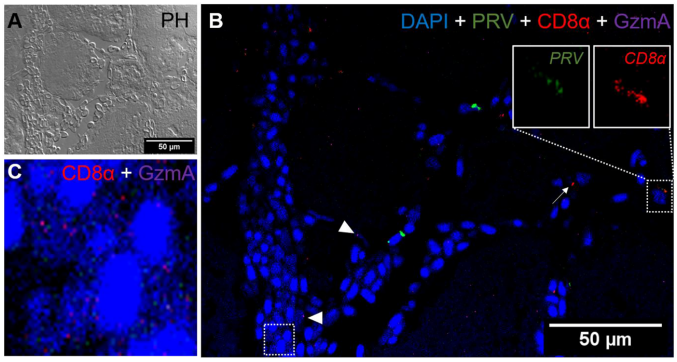

FIGURE 7 | *Fluorescent in situ hybridization of PRV-1, CD8α and GzmA in red focal changes.* **(A)** Phase contrast image showing a bleeding area with a large aggregation of blood cells. **(B)** Merged image of PRV (green), CD8α (red) and GzmA (purple). Localization of PRV-1 in CD8+ cell (dotted rectangle at right top) and co-expression of granzyme A in $CD8^+$ T cells (dotted rectangle in left bottom). Individual T cells detected expressing granzyme A specific transcripts (arrowhead) along with other CD8 cells (arrow). Nuclei DNA stained with DAPI (blue) **(C)** Magnified image of CD8+ and GzmA co-expression from dotted square in image **(B)** Scale Bar = 50 µm.

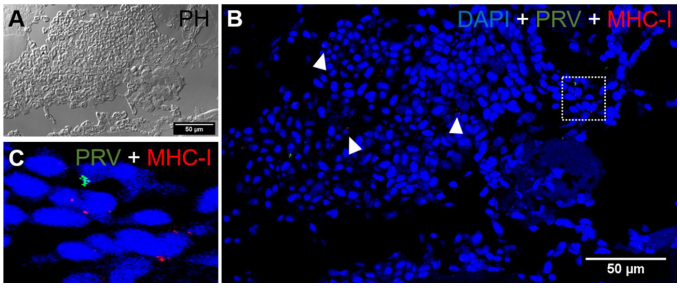

FIGURE 8 | *Fluorescent in situ hybridization of PRV-1, and MHC-I in areas of red focal changes.* **(A)** Phase contrast image showing a large hemorrhage. **(B)** Merged image of PRV-1 and MHC-I showed no co-expression of PRV-1 in MHC-I cells, but a few cells were detected in the bleeding area (arrowhead). **(C)** Magnified image from image B (dotted rectangle) showing PRV-1 infected cells along with MHC-I cells. Scale Bar = 50 µm.

the MHC-I expression level was relatively higher in black focal changes (14 folds, MWU value = 0, n1 = 4, n2 = 6, p-value = 0.0048) than in red focal changes (9 folds, MWU value = 2, n1 = 4, n2 = 5, p-value = 0.0317) (**Figure 11B**).

DISCUSSION

This study aimed to clarify the role of PRV-1 infection and the immune mechanisms involved in the development of melanized foci in white muscle of Atlantic salmon, using immune cell gene markers representing the macrophage polarization pattern, and the cytotoxic immune response.

Macrophages respond to their environment by differentiating into the functional pro-inflammatory phenotypes M1 macrophages, implicated in initiating and sustaining inflammation, or the anti-inflammatory M2 macrophages, implicated in tissue repair (33). In samples from red and black focal changes from non-infected fish there were no obvious detection of macrophage polarization apart from minimal occurrence of M2 macrophages, based on Arg2 transcript detection. Unaffected muscle areas of non-infected fish showed no presence of polarized macrophage markers. These findings were also reflected in the qPCR transcript analysis, which mirrored the *in situ* findings.

On the other hand, our results indicate that in the PRV-1 infected fish, the initial phase of the progress of the red and black spot formation were tightly connected to macrophage polarization and linked to the presence of PRV-1. The development of melanized focal changes is considered to be multifactorial. Viral diseases such as pancreas disease (PD) may affect the white muscle, but

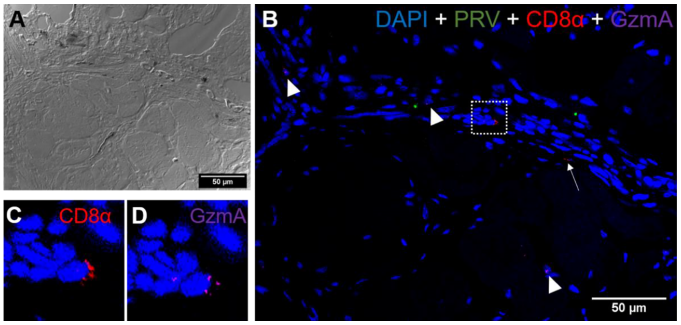

FIGURE 9 | *Fluorescent in situ hybridization of PRV-1, CD8α and GzmA in black focal changes.* **(A)** phase contrast image showing cell structures with melanin accumulation. **(B)** Merged image showing presence of PRV-1 (green) infected cells with $CD8^+$ cells (red) (arrows) and granzyme A (purple) in another cell population (arrowhead). Dotted rectangle showing co-expression of CD8 and GrzmA split in **(C, D)**. Nuclei DNA stained with DAPI (blue) Scale Bar = 50 µm.

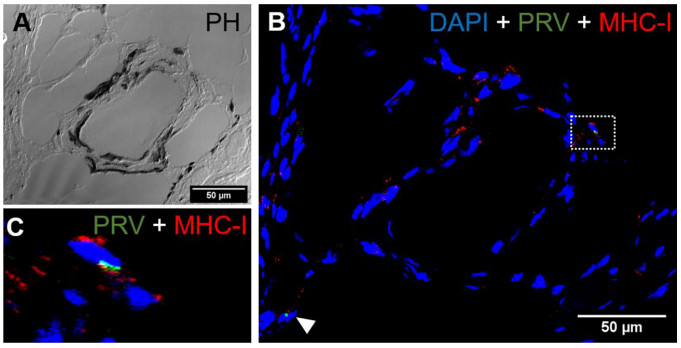

FIGURE 10 | *Fluorescent in situ hybridization of PRV-1 and MHC-I in black focal changes*. **(A)** phase contrast image showing vacuolar area surrounded by melano-macrophages and other immune cells. **(B)** Merged image showing presence of numerous MHC-I positive cells (red) where some are co-staining with PRV-1 (green) (dotted rectangle and arrowhead). **(C)** Magnified area from image B showing co-localization of PRV-1 in some MHC-I cells. Scale Bar = 50 µm.

widespread presence of melanized changes in areas being free from PD historically suggest no etiological role for this disease in the development of melanized focal changes (34). Furthermore, melanized changes have not been found to be influenced by bacterial components (2). We found that local PRV-1 infection was associated with the M1 polarized cell marker iNOS2 in the early developmental phases of melanized foci. PRV-1 was detected in a limited number of erythrocytes in hemorrhages, and only a few M1 macrophages was detected in the initial phase of red spots. Erythrocytes are a primary cell target of PRV-1 both in the acute and persistent phases of infection (7), and infected cells can be detected in any vascularized tissue. We have not found evidence here indicating that the PRV-1 infection initiated these hemorrhages as in previous studies (2, 4). However, for the close PRV relative, Grass carp reovirus (GCRV), it is suggested that iNOS2 activity is implicated in apoptosis of the vascular endothelial cells in hemorrhages characteristic for GCRV infection of Grass carp (*Ctenopharyngodon idella*) (35).

In PRV-1 infected fish, the M1 type macrophages were modestly detected in the early phase of red focal changes. However, in the more developed, intermediate phase of red spots the M1 macrophages were a dominating feature and were almost uniformly positive for PRV-1 specific staining, i.e. PRV-1 infected. The expression analysis by RT-qPCR also demonstrated elevated expression of iNOS2 in this phase. The dominating presence of PRV-1 infected, M1 polarized macrophages in this phase indicates a pro-inflammatory environment, which may be driven by the PRV-1 infection. In an earlier study, a significant downregulation of the anti-inflammatory cytokine IL10 was found associated with red changes (12), which indirectly indicates a pro-inflammatory environment.

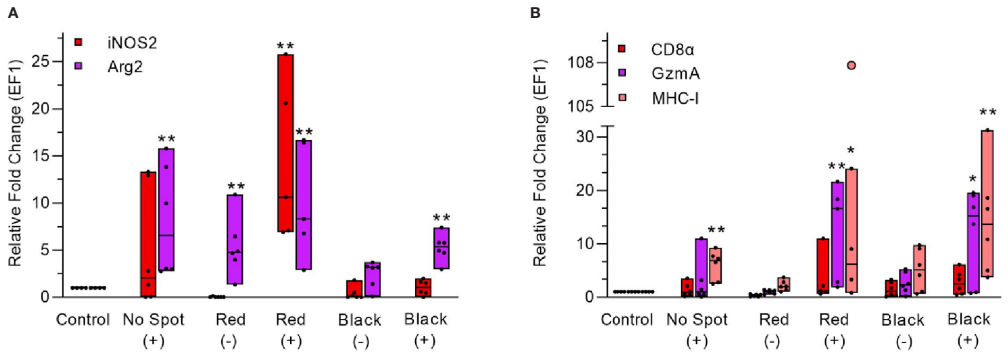

FIGURE 11 | Gene expression analysis during red and black focal changes in PRV infected (+) and uninfected (-) fish groups. **(A)** Relative fold change (median line) for each fish group is shown for iNOS2 and Arg2 (left Y-axis). **(B)** Relative fold change (median line) for each fish group is shown for CD8α, GzmA and MHC-I. Pink dot showing outlier value in the respective group. Each dot within boxes represents individual fish in the group. Gene expression relative to the control group (uninfected and unaffected) fish was normalized against EF1ab. * indicates significantly different from the control group (*$p < 0.05$, **$p < 0.01$).

Red spots with sporadic appearance of melano-macrophages were categorized as late red spot phase. This phase is considered to reflect the transition phase between red and black spots. Upregulation of iNOS2 level during red spots could be an indicator for commencement of melanogenesis. It is noteworthy that iNOS2 contributes to the melanogenesis in mammalian melanocytes (36). Based on the expression of the M2 marker Arg2, we found that the melano-macrophages at the site displayed the properties of anti-inflammatory M2 macrophages. We also found M2 macrophages without melanin. Co-localization pattern revealed PRV-1 abundance in melanized cells. The melano-macrophages of teleost fish are phagocytic cells (37) and they accumulate at long-term antigen retention sites in salmonids (13, 38). Phagocytosis of virus infected cells by macrophage and melano-macrophages have been reported earlier in Atlantic salmon (39). M2 macrophages are cells normally involved in tissue repair, and here they appeared first when melanin started to accumulate in the spots in skeletal muscle tissue.

Our findings indicate that PRV-1 infected macrophages are not innocent bystanders but represent M1 polarized macrophages important in the development of the pro-inflammatory microenvironment of red spots. The melanin accumulation starts in the late phase of red focal changes and will ultimately progress into black focal changes. It therefore seems as if melano-macrophages do not infiltrate the changes as such, but rather as non-pigmented macrophages capable of accumulating melanin over time. Melanogenesis has previously been demonstrated in advanced black spots (3). This putative progression could also be an explanation for the low prevalence of red spots but an increasing prevalence of black spots through the production period in seawater (2).

The black spots demonstrated a more heterogenous macrophage populations, i.e. both M1 and M2 macrophages were present. In advanced melanized areas, few M1 macrophages were positive for PRV-1, whereas PRV-1 co-localization was detected both in melanized (melano-macrophages) and non-melanized M2 type macrophages. In mammals, Arg2 is shown to downregulate the nitric oxide production of the M1 macrophages (40). Our findings indicate that Arg2 specific transcripts are mostly linked to the melanized area and associate with melano-macrophages. Presence of melano-macrophages (M2) was consistent from the late phase of red spots into black spots transformation (**Table 4**).

The correlated upregulation of Arg2 transcripts with the stage of development of the spots in the PRV-1 infected fish indicated a gradual shift from an inflammatory to a healing response

TABLE 4 | Consolidated summary of results.

Type of spot		*Key In situ* findings	Characteristic gene expression level
Red spot	Early	Few M1 macrophages in PRV-1 positive hemorrhages.	Significant upregulation of *iNOS2* expression
	Intermediate	High co-localization of PRV-1 in M1 macrophages.	Significant upregulation of *MHC-I* and *GzmA* expression
	Late	Detection of few M2 melano-macrophages.	
Black spot		Domination of M2 melano-macrophages and co-localization with PRV-1.	Significant upregulation of *Arg2* transcription.

during the transition from red to black macroscopic appearance of the spots. The spots of the non-infected fish with lack of detection of M1 macrophage marker and only a few detected M2 polarized macrophages, strongly indicated that PRV-1 is driving macrophage polarization in the spots of infected fish.

The initial etiological cause(s) of the red spots is unknown. The outcome of the spots in uninfected fish groups is also unknown due to the ubiquitous presence of PRV-1 in farmed Atlantic salmon, and the lack of an experimental model for spot formation (11). However, it could be speculated if the lack of inflammation in spots in non-infected fish argues for a shorter longevity and lower severity of the spots.

To further characterize the inflammatory microenvironment in the spots, the presence of CD8, Granzyme A and MHC-I positive cells was characterized during spot development. There were substantial variations in the presence of these markers among the individual fish, but *in situ* visualization indicated that MHC-I positive, PRV-1 infected cells were targeted by CD8 positive T cells both in red and black spots. The relative low number of CD8 positive cells evenly observed both in red and black focal changes was in line with the RT-qPCR expression analysis. However, a moderate, but not statistically significant up-regulation of CD8α expression in black focal changes compared to red focal changes was observed, and has been reported earlier (12). Mature cytotoxic T cells can use granzyme A for killing of target cells containing intracellular pathogens. Here, granzyme A specific transcripts were observed in cells that were not expressing CD8. By RT-qPCR, expression of Granzyme A was found to be significantly increased in both red and black spots compared to control samples, while CD8 was not. Granzyme A is also be synthesized by natural killer cells (NK-cells) (41) or other immune and non-immune cells in the teleost fish (42).

Mammalian myopathies are often marked by up-regulation of MHC-I (43, 44). By immunolabelling, MHC-I positive cells have earlier been demonstrated to be abundant in red spots (12), and in the present *in situ* study MHC-I cells were common, but perhaps not abundant. In both studies an absence of MHC-I positive myocytes was observed in the affected area, combined with lack of observation of PRV-1. The present study did not indicate that infection of the skeletal muscle cells is an important factor of the spot formation. As for Granzyme A, the MHC-I expression was significantly increased in both PRV-1 infected red and black spots compared to control samples, and co-localization of MHC-I and PRV-1 were seen in some cells especially in the melanized areas. Taken together, the targeted cell mediated immune response by the host tries to resolve and eradicate PRV-1 infection during red and black spots formation.

CONCLUSION

A possible course of events in the pathogenesis of black spots, is that PRV-1 infected erythrocytes in the hemorrhages infect tissue macrophages through phagocytosis. The myocyte degeneration in red muscle caused by PRV-1 (6), could be an additional driver for influx of macrophages, but is probably not the initial cause of red spots, as these are found at similar prevalence prior to PRV infection (2). The iNOS2 expressing M1-polarized non-melanized macrophages are mainly present in the period of the red focal changes, i.e. the time of inflammation, which suggests local production of NO and other oxygen radicals by the M1 macrophages. Melanin is a protector against free oxygen radicals (45), and its accumulation could be a consequence of the pro-inflammatory environment. Melanogenesis has previously been demonstrated in a salmon macrophage-like cell line (46, 47). The increased prevalence and severity of the black spots over time, indicates that the spot forming process is long lasting. The numerous M2-polarized melano-macrophages in black spots indicate that this is a healing phase of the process. Moreover, the presence of cytotoxic T cells and MHC-I positive cells in the focal changes represents the host' ability to target and eliminate PRV-1 infected cells. This suggests a role of PRV-1 infection in driving the development of black spots in white muscle of Atlantic salmon.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the material was achieved from commercial production.

AUTHOR CONTRIBUTIONS

Conceptualization, MM, HB and ER. Methodology, MM, IN and HB. Software, MM. Validation, MM, ØW and MD. Formal analysis, MM, HB, ØW and ER. Investigation, MM, HB, ØW and ER. Resources, ER and EK. Data curation, MM, IN. Writing—original draft preparation, MM and ER. Writing—review and editing, ØW, MM, MD, HB, EK and ER. Visualization, MM, IN and HB. Supervision, ER, EK, MD and ØW. Project administration, ER. Funding acquisition, ER and EK. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.664624/full#supplementary-material

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