Norwegian University of Life Sciences
Faculty of Veterinary Medicine

Philosophiae Doctor (PhD)
Thesis 2023:79

Inflammatory conditions in the musculoskeletal system of salmonids in Norwegian aquaculture in relation to melanized changes

Inflammasjonstilstander i muskel- og skjelettsystemet hos salmonider i norsk akvakultur relatert til melaniserte forandringer

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precisely what I needed to hear when approaching the submission of my thesis: "*Victory belongs to the most tenacious*."

1 ABBREVIATIONS

APC - antigen-presenting cell

aSVF - adipose stromal vascular fraction

CT – computed tomography

Ct - cycle threshold

DAMP - damage associated molecular pattern

DHA- docosahexaenoic acid

DMD - Duchenne muscular dystrophy

DNA - deoxyribonucleic acid

EDTA – ethylenediaminetetraacetate

EGC - eosinophilic granule cell

EPA - eicosapentaenoic acid

FAP - fibro-adipogenic progenitor cell

FM - Fontana MassonFO – fish oil

GC - germinal centers

GS - genomic selection

HE - hematoxylin and eosin

HSMB – hjerte- og skjelettmuskelbetennelese

HSMI – Heart and skeletal muscle inflammation

i.p. – intraperitoneal

Ig – immunoglobulins

iNOS - inducible nitric oxide synthase

IPNV - Infectious pancreatic necrosis virus

ISH - In situ hybridization

kV - kilo voltage

LC-PUFA – long-chain- polyunsaturated fatty acid

mA – milliampere

MAS - marker-assisted selection

mAs - milliampere/second

MFC – melanized focal changes

MHC - major histocompatibility complex

MMC - melano-macrophage centers

MGC – multinucleated giant cell

MSB - Maritus Scarlet Blue

MT - Masson Trichrome

NCC - non-specific cytotoxic cell

NET - neutrophil extracellular trap

NK-cell - natural killer cell

NO - nitric oxide

NOD- nucleotide oligomerization domain

NOFIMA - The Norwegian Institute of Food, Fisheries and Aquaculture Research

NOK – Norwegian krone

OI - osteogenesis imperfecta

OM – osteomalacia

OP – osteoporosis

PAMP - pathogen-associated molecular pattern

PD – Pancreas Disease

PRR - pattern-recognizing receptor

PRV-1/3 – Piscine orthoreovirus 1/3

QTL - quantitative trait loci

RFC – red focal changes

RIG- I- retinoic acid-inducible gene I

RNA – messenger ribonucleic acid

RNA - ribonucleic acid

ROS - reactive oxygen species

RT-qPCR - Reverse transcription quantitative polymerase chain reaction

SAV- Salmonid alphavirus

SD - sleeping disease

SHK – salmon head kidney

THK - tilapia head kidney

TLR - toll-like receptor

TNFα - tumor necrosis factor α

TRAP - tartrate-resistant acid phosphatase

Tyrp-1/2 - tyrosine related protein 1/2

VG - Van Gieson

VO – vegetable oil

2 LIST OF PAPERS

Paper I:

Anatomical and pathological characteristics of ribs in the Atlantic salmon (*Salmo salar* L.) and its relevance to soft tissue changes

Malin Brimsholm, Per Gunnar Fjelldal, Tom Hansen, Cathrine Trangerud, Geir Magne Knutsen, Charlotte Finstad Asserson, Erling Olaf Koppang, Håvard Bjørgen

Published in *Anatomia Histologia Embryologia,* 2023, *52*(3), 421-436, DOI: 10.1111/ahe.12900

Paper II (Short communication):

Diffuse melanization of the red skeletal musculature in farmed Atlantic salmon (*Salmo salar* L.)

Malin Brimsholm, Line Rønning, Espen Rimstad, Erling Olaf Koppang, Håvard Bjørgen

Published in *Journal of Fish Diseases,* 2022, *46*(4), 453-458, DOI: 10.1111/jfd.13729

Paper III:

Red and melanized focal changes in white skeletal muscle in Atlantic salmon (*Salmo salar*): Comparative analysis of farmed, wild and hybrid fish reared under identical conditions.

Malin Brimsholm, Per Gunnar Fjelldal, Tom Hansen, Thomas William Kenneth Fraser, Monica Solberg, Kevin Glover, Erling Olaf Koppang, Håvard Bjørgen

Published in *Journal of Fish Diseases*, 2023, Online version of Record, DOI: 10.1111/jfd.13856

Paper IV:

Focal red and melanized changes in the white skeletal muscle of farmed rainbow trout (*Oncorhynchus mykiss*)

Håvard Bjørgen, **Malin Brimsholm**, Morten Lund, Maria Dahle, Espen Rimstad and Erling Olaf Koppang

Manuscript

3 SUMMARY

Norwegian salmonid aquaculture has become one of Norway's largest industries. With a yearly export of 1.25 million tons, Norway is the largest producer of Atlantic salmon worldwide. Norway also has a significant production of farmed rainbow trout. The production procedures for the two species are relatively similar, with both freshwater and seawater phases due to the anadromous lifestyles of the species. During the last forty years, production has developed into a highly intensified industry, with a production time frame of about two years. To accommodate the rapid production, several improvements in fish health have emerged. An important part of the production is the selective breeding of fast-growing, slow-maturing individuals with increased robustness against diseases. Additionally, the development of efficient vaccines against several infectious agents has resulted in a significant increase in fish health and welfare and a decrease in the use of antibiotics to treat bacterial diseases. The impact of nutritional contents in fish feeds, such as lipid sources and minerals, has been given increasing attention over the last decades, as the feed can severely impact fish growth and general health.

While the aquaculture industry has made significant strides in addressing potential hazards impacting farmed fish, some challenges continue to impact fish health and welfare. An important quality concern for the salmon industry is the occurrence of red and melanized changes in the skeletal muscle, commonly known as red focal changes (RFC) or melanized focal changes (MFC). These changes are predominantly detected in the abdominal wall's cranioventral region at slaughter; on average, 20-30 % of farmed Atlantic salmon are affected. The finding of RFC and MFC leads to cassation of the affected area in the fillet, costing the industry great economic losses each year. As these changes cannot be detected before slaughter, i.e., no detectable symptoms, they are primarily considered a quality concern; however, RFC and MFC cause significant tissue damage in the white muscle and could thus be considered an animal welfare problem.

RFC and MFC represent acute and chronic inflammatory reactions in the skeletal muscle. Histological properties of RFC include hemorrhages and necrotic myocytes, while MFC is characterized by granulomatous inflammation, extensive fibrosis, and an abundance of melano-macrophages, the latter being responsible for the dark pigmentation of the fillet. The development from acute to chronic inflammation is suggested to result from persistent infection with *piscine orthoreovirus* 1 (PRV-1), as the virus has been found in relation to severe MFC and particularly to organized granulomas in the lesioned tissue. However, there is no indication that PRV-1 is the causative factor for the development of RFC, and the initial cause of the acute inflammation is not yet known. The specific localization of the changes has led to several theories on initiating factors causing focal hemorrhage and necrosis in the skeletal muscle, such as intraperitoneal (i.p.) vaccine injections, external trauma causing bruises to the muscle or inflicting fractures on the ribs, or extension of the stomach due to extensive feeding with pellets causing pressure on the abdominal wall. Some production parameters have proved to reduce the prevalence of RFC and MFC, such as increased fish oil content in the feeds, consequently increasing the level of the anti-inflammatory fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

RFC and MFC have not been observed in wild-living Atlantic salmon, and the prevalence of RFC and MFC in farmed rainbow trout and in organically farmed Atlantic salmon are believed to be lower than in common Atlantic salmon production. This has led to the question of whether the prevalence in conventional Atlantic salmon farming is due to the external factors revolving around the production

procedures or if the internal (genetic) dissimilarities between farmed and wild salmon and between salmon and rainbow trout can have an impact on the development of RFC and MFC.

This thesis aimed to address critical questions about RFC and MFC's origins in Norwegian salmonid production. In paper I, the association between pathological conditions in the ribs in relation to RFC and MFC was investigated, as their anatomical location could indicate involvement in the development of RFC/MFC. Here, Atlantic salmon from a commercial producer, Bremnes Seashore AS, experimentally kept fish from Matre Research station, and wild-caught spawning salmon were investigated using radiography and histology for costal abnormalities in relation to RFC and MFC. The results showed an association between costal fractures and RFC and MFC and between callus formations and MFC. However, RFC and MFC were also observed with no pathological changes in the ribs. The histological investigations described the anatomical properties of Atlantic salmon ribs, including vascularization of the ribs, giving further insight into the pathogenesis during costal fractures in salmon and the association to RFC and MFC.

Although most commonly seen as focal discoloration in the cranio-ventral abdominal wall, melanization has been observed in other parts of the skeletal muscle with lower frequency. Different locations and distributions may indicate a different cause and development. In paper II, a morphological description was conducted on diffuse melanization of red skeletal muscle in farmed Atlantic salmon, a condition rarely observed partially due to the retainment of the skin during slaughter. The red skeletal muscle is located right beneath the skin, on the lateral sides of the truncus, and makes up less than 10% of the total skeletal mass in salmonids. Red skeletal muscle's physiological properties differ from white skeletal muscle's, with smaller myocytes, abundant vascularization, and aerobic metabolism. Additionally, during disease outbreaks of heart and skeletal muscle inflammation (HSMI) or pancreas disease (PD) caused by PRV-1 and salmonid alphavirus (SAV), respectively, lesions are predominantly found in the red skeletal muscle and, to a lesser degree, in the white skeletal muscle. The results in paper II showed similar changes as in MFC in the abdominal wall, with diffuse granulomatous inflammation and extensive fibrosis and infiltration of melano-macrophages. No organized granulomas were present. Using reverse transcription quantitative polymerase chain reaction (RT-qPCR), PRV-1 was detected, with a statistically significant lower Ct value in the affected samples. The specific location of the virus in the tissue was not investigated; however, the characteristics of the lesions corresponded with a persistent infection of PRV-1, although without granulomas, as seen in advanced high-grade MFC in white skeletal muscle.

To determine whether the differing occurrence of RFC and MFC in farmed and wild Atlantic salmon arises from external influences or genetic factors, a study was conducted in which Atlantic salmon from farmed, wild, and hybrid backgrounds were raised under identical rearing conditions from hatching until slaughter (paper III). The prevalence of the changes was investigated before and after seawater transfer. In accordance with earlier reports, RFC and MFC occurred in the seawater phase. RFC remained stable at 1-5 % throughout the trial, whereas MFC increased towards slaughter, reaching a 25-30 % prevalence. There was no significant difference in prevalence across the origin groups, and histological investigations revealed similar changes in both farmed, wild, and hybrid fish. These results indicate that the changes develop due to external production-related factors and thus explain the absence of RFC and MFC in wild-living Atlantic salmon.

The variation in prevalence seen in farmed rainbow trout has also been hypothesized to result from genetic differences, especially given the similar rearing conditions shared with Atlantic salmon production. To provide novel information on the frequency and morphological characteristics of RFC and MFC in farmed rainbow trout, investigations were conducted on slaughter-ready fish from three different farm sites in Western Norway (paper IV). RFC was only detected in one fish, whereas MFC

had a maximum prevalence of 6.47%. Histological investigations revealed some similarities to those found in Atlantic salmon, however, with a lower presence of melano-macrophages and other inflammatory cells embedded in the lesioned and fibrotic tissue. Importantly, organized granulomas were only detected in one fish. PRV-1 and SAV were detected in skeletal muscle with RT-qPCR, and *in situ* hybridization (ISH) revealed that the viruses were not located in relation to the lesions, in contrast to PRV-1 in severe melanized changes in Atlantic salmon. These results indicate that the response and clearance of viruses are dissimilar and more efficient in rainbow trout, in accordance with earlier comparative reports on viral-specific responses, possibly explaining the lower prevalence of MFC in rainbow trout. Thus, the dissimilar frequency of MFC in farmed Atlantic salmon and rainbow trout is likely due to genetic divergencies between the two species. Additionally, the lower prevalence of RFC in rainbow trout indicates a lower impact of an external factor than in Atlantic salmon production, causing local hemorrhage and necrosis.

In sum, this thesis sheds light on the initial factors causing RFC and MFC in farmed Atlantic salmon and rainbow trout. The results emphasize the high significance of external factors and the low influence of genetic impact on the prevalence of focal changes in farmed Atlantic salmon and pinpoint costal fractures as one possible external factor that can cause RFC and MFC. Novel insight into the anatomical properties of Atlantic salmon ribs was revealed, providing essential information for future analyses of pathological changes in the ribs and adjacent soft tissue. Different distributions of melanization in Atlantic salmon may have different causes, such as diffuse melanization of the red skeletal muscle. However, the severity and duration of the chronic inflammation are suggested to result from the persistent infection of PRV-1, regardless of distribution. This thesis provides the first description of RFC and MFC in farmed rainbow trout. The findings imply that the lower prevalence of MFC in rainbow trout results from genetic divergencies between the two species, causing different viral defenses and elimination. Additionally, external factors may inflict less harm on farmed rainbow trout than Atlantic salmon, as the prevalence of RFC was also lower.

4 NORSK SAMMENDRAG (SUMMARY IN NORWEGIAN)

Norsk lakseproduksjon utgjør i dag en av Norges største industrier, og med en årlig eksport på 1.25 millioner tonn er Norge verdens største produsent av atlantisk laks. Norge har også en betydelig produksjon av regnbueørret. Produksjonen av disse artene er tilnærmet like, med både ferskvannsfase og sjøvannsfase, hvor utgangspunktet er de anadrome egenskapene til artene. Denne industrien har i løpet av de siste årene blitt svært intensivert, med en produksjonssyklus på rundt 2 år. Flere tilpasninger er gjort innen fiskehelse for å best kunne imøtekomme denne hurtige produksjonen. En viktig del av dette arbeidet er seleksjonsbasert avl av rasktvoksende individ med sen seksuell modning og økt robusthet mot infeksjonssykdommer. I tillegg har utviklingen av effektive vaksiner resultert i betydelig forbedret fiskehelse og velferd, og ikke minst en kraftig nedgang i bruken av antibiotika ved behandling av bakterielle sykdommer. De senere årene har også fokuset på næringskomponenter i fiskefôret økt, som mineral- og lipidinnhold, da det viser seg at fôret har stor betydning for fiskens vekst og helse.

På tross av store forbedringer innen laksens helse og velferd i oppdrettsnæringen er det fortsatt utfordringer knyttet til produksjonen. Et viktig kvalitetsproblem i næringen er forekomsten av røde og svarte flekker i den hvite skjelettmuskulaturen hos oppdrettslaks. Flekkene finnes hovedsakelig i den cranioventrale delen av bukveggen, og sees i gjennomsnitt i 20-30 % av fisken. Ved funn av slike flekker blir deler av, eller hele, fileten kassert, noe som fører til store økonomiske tap for næringen hvert år. Ettersom affisert fisk ikke viser symptomer blir ikke flekkene oppdaget før slakt, og dermed ansees dette hovedsakelig som et kvalitetsproblem. Fiskens velferd bør dog ikke ignoreres, ettersom de røde og svarte flekkene er et resultat av betydelig vevsskade i skjelettmuskulaturen.

Røde og svarte flekker representerer henholdsvis en akutt og kronisk inflammasjonsreaksjon i skjelettmuskulaturen. Histologiske forandringer i røde flekker innebærer blødning og nekrose av muskelceller, mens det i svarte flekker foregår en kronisk, granulomatøs inflammasjon med uttalt fibrose, og med betydelig infiltrasjon av melanomakrofager som gir den distinkte mørke fargen i muskelvevet. Utviklingen fra akutt til kronisk inflammasjon skyldes trolig en persistent infeksjon av *piscine orthoreovirus* 1 (PRV-1). Dette viruset er sett i relasjon til alvorlige grader av svarte flekker hos atlantisk laks, og også i organiserte graulomer i vevet. Det er derimot ingen indikasjon på at PRV-1 er den utløsende årsaken til den akutte inflammasjonen. På grunn av sin spesifikke lokalisasjon i bukveggen har flere teorier blitt foreslått som årsak til de røde, og senere svarte, flekkene. Eksempler på slike teorier er både intraperitoneal (i.p.) vaksineinjeksjon, eksternt traume som gir skade på skjelettmuskulaturen eller ribbena, eller utvidelse av magesekken grunnet uttalt fôring med pellets som gir et innvendig trykk på bukveggen. Enkelte produksjonsparametere har dessuten vist seg å redusere forekomsten av røde og svarte flekker, som økt fiskeoljeinnhold i fôret, med påfølgende økt innhold av de anti-inflammatoriske fettsyrene eicosapentaensyre (EPA) og dokosaheksaensyre (DHA).

Røde og svarte flekker er ikke observert i vill Atlantisk laks, og forekomsten av flekkene er trolig lavere i oppdrett av regnbueørret og økologisk laks. Dette har ført til spørsmålet om forekomsten av flekker hos konvensjonelt produsert laks skyldes eksterne faktorer i produksjonsprosedyrene, eller om det er interne (genetiske) forskjeller mellom oppdrettet og vill laks, og mellom atlantisk laks og regnbueørret, som er avgjørende for den ulike frekvensen av flekker.

Denne doktorgradsavhandlingen adresserer viktige spørsmål rundt opphavet til de røde og svarte flekkene i norsk laksefiskproduksjon. I artikkel I ble assosiasjonen mellom røde og svarte flekker og patologiske forandringer i ribbena undersøkt, ettersom fiskens ribben ligger i nær relasjon til bukhulen og potensielt kan føre til skade i bukhulemuskulaturen. Ved hjelp av radiografiske og

histologiske metoder ble prøver fra Atlantisk laks fra en konvensjonell oppdretter undersøkt, i tillegg til prøver fra Matre Forskningsstasjon og villfanget laks. Resultatet viste en sammenheng mellom ribbensfrakturer og røde og svarte flekker i skjelettmuskulaturen, samt en sammenheng mellom callusformasjoner i ribbena og svarte flekker. Røde og svarte flekker ble også observert uten patologiske forandringer på ribbena, som indikerte andre årsaksforhold enn ribbensforandringer. Gjennom histologiske undersøkelser ble anatomiske forhold i ribbena til Atlantisk laks beskrevet, inkludert vaskularisering av ribbena, noe som gir viktig kunnskap for patogenesen ved et ribbensbrudd, men som også understreker assosiasjonen til røde og svarte flekker.

Selv om flekkene normalt sees som en fokal misfarging i den cranioventrale bukveggen, er melanisert skjelettmuskulatur også observert i andre deler av fisken, dog mer sjeldent. Ulik lokalisasjon og distribusjon av melaniseringen peker på andre utløsende årsaker og /eller ulik utvikling av inflammasjonen enn for de fokale flekkene. Artikkel II presenterer en morfologisk beskrivelse av diffus melanisering av den røde skjelettmuskulaturen i atlantisk laks, en tilstand som sjelden observeres, delvis fordi skinnet på fisken vanligvis ikke fjernes ved slakt. Den røde skjelettmuskulaturen utgjør under 10 % av den totale skjelettmuskelmassen i laksefisk. De fysiologiske egenskapene skiller seg fra den hvite skjelettmuskulaturen, med myocytter av mindre størrelse, uttalt vaskulering og aerob metabolisme. Dessuten, under utbrudd av hjerte- og skjelettmuskelbetennelse (HSMB) og pankreas sykdom (PD), forårsaket av henholdsvis PRV-1 og *salmonid alphavirus* (SAV), er det fortrinnsvis den røde skjelettmuskulaturen som affiseres, og i mindre grad den hvite. Resultatene i artikkel II viste lignende histologiske forandringer som er beskrevet i fokale svarte flekker, med diffus granulomatøs betennelse, uttalt fibrose og melanomakrofag-infiltrasjon. Organiserte granulomer ble ikke observert. Ved bruk av teknikken reverse transcription quantitative polymerase chain reaction (RT-qPCR), ble PRV-1 detektert i muskelprøvene, med signifikant lavere Ct-verdi i de affiserte fiskene. Den spesifikke lokalisasjonen til viruset i vevet ble ikke avdekket, men lesjonenes karakteristikker korresponderte med den persistente PRV-1-infeksjonen som er vist i høygradige fokale svarte flekker.

For å avdekke om den ulike forekomsten av flekker i oppdrettslaks og villaks skyldes eksterne eller genetiske faktorer, ble det i artikkel III gjennomført en studie hvor atlantisk laks med ulike opphav (avlet, vill og hybrid) ble holdt i identiske oppdrettsforhold fra klekking til slakt. Forekomsten av flekker ble undersøkt både i ferksvannsfasen og sjøvannsfasen. I samsvar med tidlige rapporter viste resultatene at flekkene oppstod i sjøvannsvasen av produksjonen. Forekomsten av røde flekker holdt seg stabil gjennom hele sjøvannsfasen, med en prevalens på 1-5%, mens de svarte flekkene økte i prevalens gjennom sjøvannsfasen, og ved slakt lå denne på 25-30 %. Det var ingen statistisk signifikant forskjell i prevalensen mellom de ulike opphavsgruppene, og histologiske undersøkelser viste lignende forandringer i alle gruppene. Disse resultatene indikerer at flekkene i oppdrettslaks oppstår som en konsekvens av eksterne faktorer i oppdrettsnæringen, noe som forklarer fraværet av flekker i villaks.

Den ulike forekomsten av flekker hos oppdrettet regnbueørret har også ført til spekulasjoner om dette skyldes eksterne eller genetiske ulikheter mellom de to artene. Her er særlig den genetiske forskjellen foreslått, ettersom oppdrettsforholdene av artene er nærmest tilsvarende. Viktig informasjon om frekvensen og morfologien til flekker i regnbueørret ble presentert i artikkel IV, hvor slakteferdig regnbueørret fra tre ulike oppdrettsanlegg ble analysert. Røde flekker ble kun observert i én fisk, og den høyeste forekomsten av svarte flekker var 6,47%. Histologiske undersøkelser viste lignende forandringer som i atlantisk laks, men med lavere forekomst av infiltrerende melanomakrofager i det fibrotiske vevet. I tillegg ble det kun observert organiserte granulomer i en av fiskene. Både PRV-1 og SAV ble detektert i skjelettmuskelprøvene ved hjelp av RT-qPCR, og ved

bruk av *in situ* hybridisering (ISH) ble det avdekket at virusene var lokalisert i områder uten lesjoner. Dette er i kontrast til høygradige svarte flekker hos atlantisk laks, hvor PRV-1 er assosiert med affiserte områder og organiserte granulomer. Disse resultatene indikerer ar responsen mot, og elimineringen av virus er ulik og mer effektiv i regnbueørret, noe som stemmer overens med publiserte resultater omhandlende komparativ virusrespons hos laksefisk. Dette er trolig også forklaringen på den lavere forekomsten av svarte flekker hos regnbueørret. Dermed er den ulike forekomsten av svarte flekker hos atlantisk laks og regnbueørret trolig grunnet genetiske ulikheter. Den lave forekomsten av røde flekker i regnbueørret indikerer dessuten at den utløsende, eksterne årsaken til flekker er av mindre betydning enn i oppdrett av atlantisk laks.

For å konkludere omhandler denne avhandlingen utløsende faktorer for røde og svarte flekker i atlantisk laks og regnbueørret. Resultatene understreker betydningen av eksterne faktorer, og eliminerer betydningen av genetiske faktorer i forekomsten av flekker hos oppdrettslaks. Her vises det at ribbensfrakturer er en slik ekstern faktor som kan føre til utviklingen av flekker. Ny informasjon om anatomiske forhold i ribben hos atlantisk laks bidrar til viktig kunnskap ved tolkning av ribbensforandringer og patologiske endringer i det omkringliggende bløtvevet. Ulik distribusjon av melanisering i atlantisk laks kan ha ulike årsaker, som diffus melanisering av rød skjelettmuskulatur, men varigheten og alvorlighetsgraden er trolig et knyttet til en persistent infeksjon av PRV-1, uavhengig av distribusjon. Denne avhandlingen presenterer den første beskrivelsen av røde og svarte flekker i oppdrett av regnbueørret. Funnene viser at forekomsten av svarte flekker er lavere enn hos oppdrettslaks, og at dette trolig skyldes genetiske forskjeller mellom de to artene, som hovedsakelig resulterer i ulikt forsvar mot virusinfeksjon. Eksterne faktorer er trolig også av mindre betydning ved oppdrett av regnbueørret, ettersom forekomsten av røde flekker også var lavere her.

5 INTRODUCTION

5.1. Background for the thesis

5.1.1 Atlantic salmon and rainbow trout in Norwegian aquaculture

The Norwegian aquaculture production is primarily based on salmonid species, herein Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Both species are anadromous. Although the distinction between resident freshwater rainbow trout and the migrating steelhead trout has been made, they both belong to the same species. In the wild, the fertilized eggs are hatched (for Atlantic salmon in the fall, for rainbow trout in the spring), and the *parr* migrates to the sea as *smolts* after smoltification for growth and maturation. Adult spawning fish return from the sea to their native freshwater rivers (Fig. 1) (Thorstad, Whoriskey, Rikardsen & Aarestrup, 2011; Hardy, 2002). Wild Atlantic salmon can be found in large areas of the northern Atlantic Ocean, including North America, the Faroe Islands, and Norway (Thorstad et al., 2011a), and usually spend around 2 to 3 years at sea before returning to their native rivers (Wennevik & Hansen, 2022). In Norway, two relict tribes of Atlantic salmon exist; the *byglandsbleke* found in Byglandsfjorden (Barlaup, 2011), and *småbanken* in Namsenvassdraget (Thorstad et al., 2011b). Both tribes live solely in freshwater and are genetically dissimilar to their anadromous counterparts, as they were likely separated from the migrating Atlantic salmon during the ice age. In contrast to Atlantic salmon, rainbow trout belongs to the Pacific trout and salmon group and is not commonly found in the Norwegian wild fauna (Muhlfeld et al., 2019). Brown trout (*Salmo trutta*), on the other hand, is commonly found in Norwegian wild fauna. This is a desired species for recreational fishing but is not utilized in salmonid production in Norway. Although the species show similarities in terms of life cycle and breeding, Atlantic salmon and rainbow trout belong to separate monophyletic groups (Crête-Lafrenière, Weir, & Bernatchez, 2012; Stearley & Smith 1993) and therefore hold different traits in terms of phenotype and genotype. For a long time, the rainbow trout today known as *Oncorhynchus mykiss* Walbaum was named based on similarities to the genus *Salmo* and was thus known under *Salmo gairdneri* Richarson. However, due to more advanced biochemistry techniques, the more appropriate classification was given to rainbow trout as they proved to be more closely related to Pacific trouts (Smith & Stearly, 1989). Farming and production of Atlantic salmon in Norway started in the 1970s and has grown to become one of the largest salmon productions in the world. In 2022, 1.25 million tons of Atlantic salmon were exported for 100.8 billion NOK (Norwegian Seafood Council, 2023). Internationally, rainbow trout is the most common reared trout, with production often limited to freshwater dams and raceway ponds. In Norway, rainbow trout are reared similarly to Atlantic salmon, although on a smaller scale. The production in Norway expanded greatly during the 1990s (Skaala et al., 2020), and in 2022, 54,000 tons of rainbow trout were exported, with a value of 5.1 billion NOK (Norwegian Seafood Council, 2023).

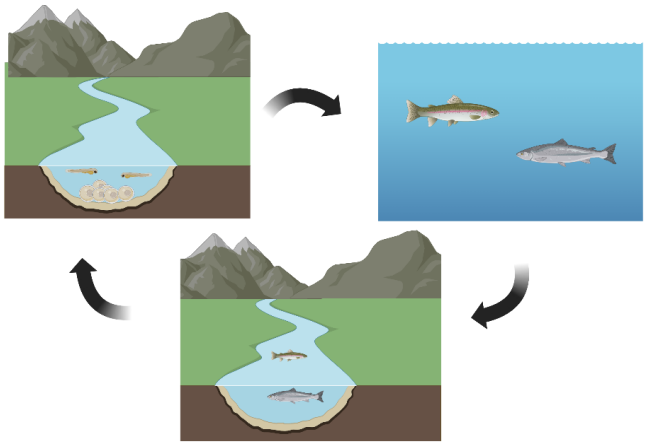

Figure 1. Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss) life cycle. Eggs hatch in freshwater rivers, alevins, fry, and later parr stay in the river until smoltification. The fish migrate to the sea as smolts and return to the native river as spawning adults. Created with BioRender.com

Production of salmonids is based on the fish's natural life cycle but with modern technology and breeding programs to accelerate growth and thus produce large quantities within a limited time. After hatching, the fish is kept in freshwater for 6 to 18 months. After sea transfer, the fish is reared for 11 to 18 months until reaching the preferred slaughter size of around 4-5 kg before sexual maturation (Fig. 2). The Norwegian breeding programs for Atlantic salmon and rainbow trout were developed in the early 70s (Gjedrem, 1992, 2010), herein selecting for beneficial traits such as late maturation, fat content and resistance against specific diseases. This meticulous work has resulted in today's farmed salmonids being genetically and phenotypically different from their wild-living counterparts (Glover et al., 2017). In the event of farmed salmonids escaping the nets, these genetic differences pose a potential threat to the genetic integrity of the wild population of Atlantic salmon and rainbow trout.

Salmonid aquaculture has several challenges in terms of production purposes and animal welfare. The fish is kept in tanks and sea cages. Throughout the production, handling of the fish is required during transportation, vaccination, and mechanical and medical treatment against disease and infections. Vaccination against several infectious agents has dramatically improved farmed fish welfare during the last decades, and antibiotics use is thus significantly reduced (Norwegian Seafood Council, 2021). However, some challenges remain to be resolved, and to ensure optimized fish health and, therefore, production, research on these topics is of great interest and importance. One significant challenge yet to be resolved is focal melanized skeletal muscle inflammation in farmed salmonids.

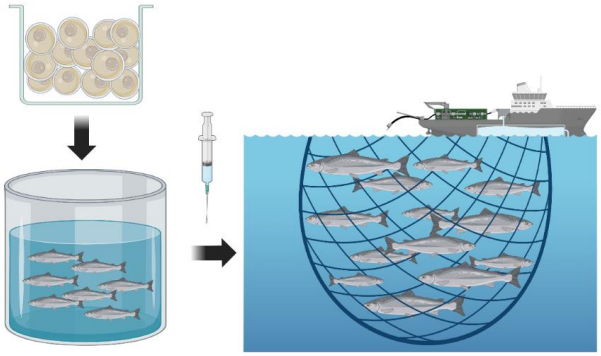

Figure 2. Simplified illustration of the Norwegian production cycle of Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss). Fertilized eggs hatch in freshwater containers. Alevins, fry, and later parr stays in freshwater tanks until smoltification. Before sea transfer, the fish is vaccinated. Transfer to seawater cages occurs post-smoltification. The fish is slaughtered before maturation when body weight reaches>2 kg. Created with BioRender.com

5.1.2 Melanization of skeletal muscle in farmed Atlantic salmon and rainbow trout

Focal red and melanized skeletal muscle are seen as red or dark discolored areas in farmed Atlantic salmon's white skeletal muscle, the fillet. The fillet is the main product retrieved from salmonid production and should appear light orange and show no sign of damage to acquire the highest quality score. At slaughter, the fillet is evaluated, and findings of discolorations lead to the cassation of parts of or the entire fillet. An average of 20 % of the fillets in farmed Atlantic salmon have melanized discolorations. Thus, the associated economic loss is substantial (Mørkøre et al., 2015). Interestingly, according to industry representatives, the prevalence of skeletal muscle melanization in rainbow trout is much lower, although the rearing procedures are similar.

The most common manifestation of melanization is found in focal areas in the cranioventral part of the skeletal muscle (90 % of the changes are found here) (Bjørgen et al., 2019). The melanized changes result from local chronic inflammation in the muscle (Koppang, Haugarvoll, Hordvik, Aune, & Poppe, 2005; Larsen et al., 2012). The condition commences with an initial injury of unknown cause, seen as hemorrhage and necrosis in the soft tissue, appearing as a red focal change (RFC) in the fillet. The leading theory is that acute inflammation can develop into a chronic state over time, resulting in a melanized focal change (MFC), possibly due to a persistent infection. Previous research strongly suggests a possible link between *piscine orthoreovirus* 1 (PRV-1) and melanized focal changes (Bjørgen et al., 2019; Bjørgen et al.,2020; Bjørgen et al., 2015; Malik et al., 2021a). However, although it is believed that PRV-1 is an important driver of the inflammation from acute to chronic, the initial cause of the acute inflammation is still unknown. Several theories have been suggested, such as trauma, hypoxia, nutritional-related factors, and genetic predispositions. As it appears, the cause of the development is multi-factorial, and it is difficult to distinguish between the different factors as there is no available model for the induction of melanized changes.

5.2 The musculoskeletal system in salmonids

In vertebrates, the musculoskeletal system constitutes the skeleton and the attached soft tissue, enabling movement of the organism. The soft tissues include skeletal muscle, ligaments, and tendons. Interspersed between the muscle tissue are adipose tissue, nerves, and blood vessels, enabling the recruitment of local energy sources and cells from the circulation. In the following sections, emphasis is given to the tissues of the musculoskeletal system in salmonids.

5.2.1 Bone tissue

Of the 34,000 fish species known, 28,000 are classified as bony fish (teleosts). The teleost skeleton consists primarily of bony tissue, unlike cartilaginous species such as sharks and rays, with a skeleton made of cartilage. However, within the teleostean species, there is a large variety of phenotypical and cellular traits of the cartilage and osseous tissue depending on the skeletal element, species, environmental conditions, and other internal and external factors (Witten, Huysseune, & Hall, 2010). The skeleton in teleost species enables swimming movement and protects the brain, spinal cord, and other internal organs. It can be divided into the *axial skeleton*; the skull and its skeletal elements, the vertebral bodies and their extensions and the ribs and intermuscular bones, and the *appendicular skeleton*; the fins, and the pectoral and pelvic girdle. In salmonids, vertebral bodies vary in number from 45 to 65 (Kacem, Meunier, & Baglinière, 1998; Kryvi & Poppe, 2016). Extending from the vertebral bodies in the thoracic region are the neural arches and spines (dorsal) and the ribs (ventral) (De Clercq et al., 2017). Haemal arches and spines (ventral) replace the ribs in the caudal part of the fish posterior to the abdominal cavity (Fig. 3). Not to be mistaken with the ribs, the intermuscular bones, known as *pin bones,* are attached to the vertebral bodies via ligaments (Kague et al., 2019). The presence of intermuscular bones varies among species and can be numerous (Patterson & Johnson 1995). At slaughter, the axial and appendicular skeleton is mechanically removed, leaving the skeletal muscle, the *fillet*, as the main product for human consumption.

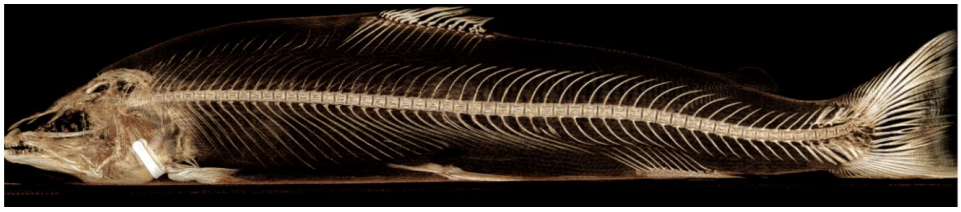

Figure 3: 3D computed tomography (CT) image of the Atlantic salmon skeleton, including the axial and appendicular skeletal elements. Imaging done by Cathrine Trangerud.

Skeletal abnormalities in salmonid aquaculture can have significant consequences for fish welfare and health but can also affect the fillet's quality. Vertebral deformities such as lordosis, kyphosis, or fused vertebral bodies may leave the fish morphology altered and impact swimming movement and feeding, and has thus been given great interest (Fjelldal et al., 2012; Gil Martens et al., 2010; Sullivan, Hammond, Roberts, & Manchester, 2007). Some focus has also been given to the ribs, predominantly in terms of anatomical deviations in relation to phosphorus deficiency but also other factors influencing normal morphology (Baeverfjord, Asgard, & Shearer, 1998; Deschamps, Stewart,

Demanche, & Vandenberg, 2016; Gislason et al., 2010; Jiménez-Guerrero et al., 2022). However, as rib malformations in salmonids have less impact on the fish's growth, performance, and external appearance, the ribs are not investigated and described to the same extent.

In Atlantic salmon, the number of paired ribs is between 21 and 24 (Jiménez-Guerrero et al., 2022), extending from the vertebral bodies reaching toward the ventral part of the abdominal wall, providing both protection for the abdominal organs (Fig 4a), and muscle attachment sites for the skeletal muscle. As salmonids lack a sternum, the ribs are not anchored ventrally but end blindly in the ventral part of the abdominal wall (Fig.4b). The ribs are located beneath the peritoneum, near the abdominal cavity (Fig.4c). Due to high breaking force (Fiedler, Elmogazy, Courtemanche, Cardoso, & Berteau, 2021) they withstand the force generated by the attached skeletal muscle during swimming. The ribs are thinner in rainbow trout but have greater breaking force than in Atlantic salmon (Yao, 2017). The axis of the ribs can be divided into proximal, mid, and distal parts (Jiménez-Guerrero et al., 2022; Soliman, 2018) and is made up of a bone collar of cortical bone covering a central medullar cavity. In mammals, the ribs are flat bones developing through type I endochondral ossification, resulting in a marrow cavity with spongious bone and hematopoietic tissue. In contrast, salmonid ribs are formed through perichondral ossification and type II endochondral ossification (explained in detail in later sections) (De Clercq et al., 2017; Weigele & Franz-Odendaal, 2016). Here, cartilage is replaced with adipose tissue and vessels. Thus, in contrast to mammals, hematopoiesis in teleosts occurs in the hematopoietic tissue of the kidney, predominantly the head kidney (reviewed by (Bjørgen & Koppang, 2021). However, the chondrocytic core in the salmonid rib can persist, and the cartilage resorption is not related to a specific time of skeletal development Witten & Huysseune, 2009). To further elaborate on the ossification process in salmonid ribs, the microstructure and cells of the bone tissue are described in the following section.

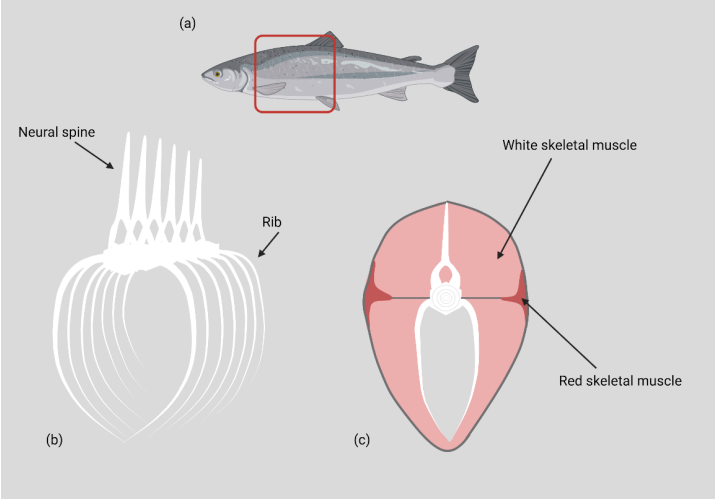

Figure 4. Illustration of the anatomical position of the ribs in salmonids. (a) The approximal region of the fish axis containing ribs. (b) Ribs extending ventrally from the vertebral bodies. Neural arches and spines extend dorsally. (c) The ribs are situated in the abdominal wall attached to white skeletal muscle tissue. Red skeletal muscle tissue is located on the lateral side of the truncus. Created with BioRender.com.

The microstructure and cells of teleost bones are used in classifying fish species as primitive/basal or advanced species. This is putatively due to the evolutionary alterations in the components of bone. The bone tissue in salmonids is characterized as primitive, with osteocytes embedded in the bone matrix consisting of type 1-collagen and hydroxyapatite crystals, primarily consisting of calcium (Ca) and phosphorus (P) (Witten et al., 2019), as seen in mammals. Thus, the term cellular bone is applied. In contrast, advanced teleosts lack embedded osteocytes in the bone tissue, and their bones are known as acellular bone (Cohen et al., 2012; Davesne et al., 2019; Horton & Summers, 2009). In vertebrates, including mammals and fish, bone cells, i.e., osteoblasts, osteocytes, and osteoclasts, are vital in the bone's growth, adaptation, and plasticity. As in mammals, teleost osteoblasts lay down the bone matrix, and when surrounded by calcified bone, the cells are characterized as osteocytes located in lacunas. Osteocytes are essential for the homeostasis and plasticity of the bone, and adjacent osteocytes communicate through canaliculi holding cell extensions (Schaffler, Cheung, Majeska, & Kennedy, 2014). Additionally, osteocytic osteolysis has been suggested as an essential trait in cellular species, including salmonids, to control mineral homeostasis (Kacem et al., 1998). Osteoblasts and osteocytes are of mesenchymal lineage in mammals (Mizoguchi & Ono, 2021) and fish (Ando, Shibata, Has, Brand, & Kawakami, 2017). Osteoclasts remove old and damaged bone through resorption activity during growth, development, and repair. Osteoclasts have also been referred to as chondroclasts during cartilage removal (Odgren, Witwicka, & Reyes-Gutierrez, 2016; Wlodarski, Brodzikowska, & Kuzaka, 2014). For convenience, this thesis refers to cells resorbing bone and cartilage as osteoclasts. As in mammals, teleost osteoclasts are thought to be of hematopoietic lineage (Persson, Björnsson, & Takagi, 1999; Takagi & Kaneko, 1995). In zebrafish, two types of osteoclasts have been detected during growth and remodeling using tartrate-resistant acid phosphatase (TRAP) staining. The two osteoclast types appear age- and site-specific; Mononucleated osteoclasts with shallow resorption lacunas are observed in younger animals, while multinucleated cells creating resorption larger lacunas (Howships lacunae) are predominantly seen in older animals (Persson et al., 1999; Witten, Hansen, & Hall, 2001). Further, during fracture repair, two types of osteoclasts are recruited to the site of damage, as seen in medaka (Takeyama, Chatani, Takano, & Kudo, 2014). The process of fracture repair in teleosts is addressed later in the thesis.

Teleosts possess an indeterminate growth, meaning the body size will grow throughout their life span (Froehlich, Fowler, Galt, Smith, & Biga, 2013). To sustain the growth and plasticity in the skeleton, the bone tissue is constantly changing through bone formation by osteoblasts and bone resorption by osteoclasts. Several factors can influence bone remodelling in addition to growth, such as mechanical impact, mineral homeostasis, metabolic demands, infectious agents, and fracture repair. The resorption of bone tissue also allows blood vessels to penetrate the bone, resulting in the vascularization of the skeletal element. Depending on fish size, skeletal element, and cellularity, vascularization of teleost bones varies significantly among species and even among individuals (Moss, 1963). Different skeletal elements may be classified as vascular or avascular, and cavities containing blood vessels in the bone tissue are known as *vascular cavities* or *vascular canals* (Meunier, 2002). The vascular canals are categorized according to organization along the axis of the bone and can be divided into radial, longitudinal, or circular vascular canals (Meunier, 2002). In mammalian bone, longitudinal vascular canals are often found in organized osteons, or Haversian systems, with osteocytes embedded in concentric bone lamellae surrounding longitudinal vessels and nerves. Transverse vessels known as Volkmann's canals provide communication between osteons. Osteons are present in compact bone and spongy bone. In teleosts, osteons can be found but may also be absent. Additionally, the presence of embedded osteocytes in the concentric lamella is primarily found in larger species (Nicoletta et al., 2015; Weigele & Franz-Odendaal, 2016).

According to Meunier (2002), vascularization in fish bone is primarily found in the inner spongy bone, whereas the outer compact bone is generally poorly vascularized. However, as shown in the dorsal fin of Atlantic bluefin tuna, an inner layer of the compact bone holds abundant osteons with central vascular canals (Santamaria et al., 2018). As for salmonid ribs, no extensive research has been done specifically on vascularization. However, cellular and acellular fish ribs have revealed vascular canals within osteons (Davesne et al., 2019). On the contrary, ribs without abundant vascularization have been observed in both primitive and advanced species (Cohen et al., 2012), reaffirming the findings done by Moss on the diversity of teleost skeletal vascularization (Moss, 1963). Nevertheless, vascularization is a vital part of the formation of bony tissues, the osteogenesis.

The osteogenesis in teleosts occurs primarily through perichondral, endochondral, and intermembranous ossification, depending on the skeletal element and the species in question. Perichondral and endochondral ossification requires a pre-existing template of cartilage tissue, making this an indirect type of ossification. In perichondral ossification (fig. 5a), osteoprogenitor cells in the perichondrium differentiate into osteocytes, laying down the bone matrix. Thus, perichondrium is transformed into periosteum, and a collar of compact bone surrounds the cartilage template. By perforating blood vessels, osteoclasts and osteoblasts will gain access to the chondrocytic medulla. In mammals, osteoclasts delivered by the perforating blood vessels remove the calcified cartilage, and osteoblasts lay down osteoid, resulting in the cancellous bone. This process, where cartilage is replaced by cancellous bone inside the bone collar, is known as endochondral ossification type I (Fig. 5b). In teleosts, osteoclasts remove the chondrocytes, resulting in a medullar cavity filled with adipose tissue (Giovannone et al., 2019), but without the presence of cancellous bone or hematopoietic tissue. This type of ossification is named endochondral ossification type II (Fig.5.c) (Weigele & Franz-Odendaal, 2016). Intramembranous ossification is a form of direct ossification through the differentiation of mesenchymal stem cells to a condensed area of osteoblasts (Fig 5d). The osteoblasts lay down bone matrix and are later enclosed by the calcified bone as osteocytes, whereas mesenchymal stem cells continue to differentiate, providing additional osteoblasts for bone matrix production. Intramembranous ossification can be found in several skeletal elements in vertebrates and is the most common type of ossification in flat bones such as the bones of the skull in mammals or the intermuscular bones in teleosts (Nie et al., 2019).

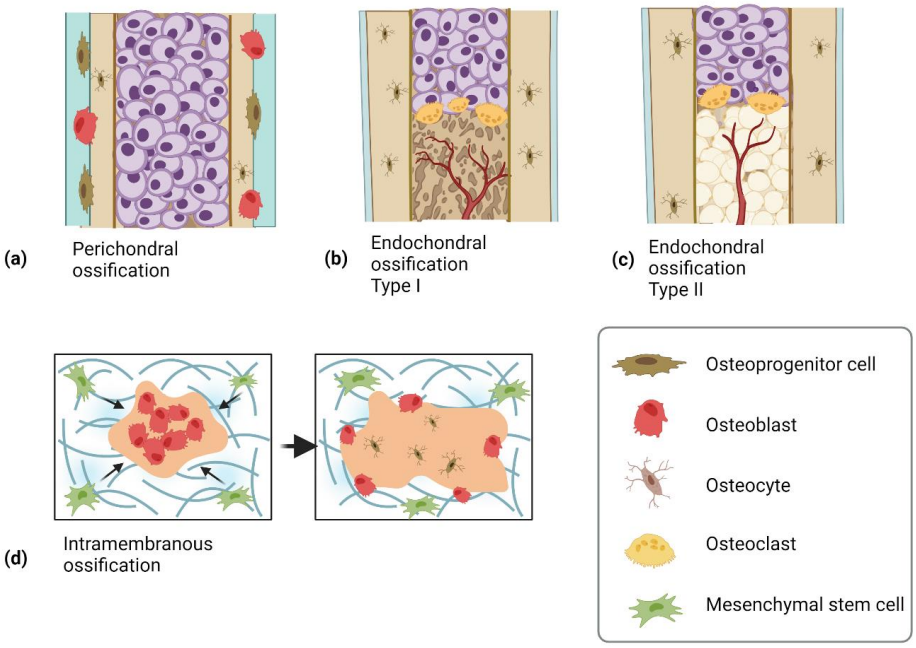

Figure 5. Illustration of ossification models. (a) Perichondral ossification, resulting in a bone collar of compact bone holding a marrow cavity. (b) Endochondral ossification type I: Perforating blood vessels supplying cells for removal of chondrocytes and deposition of bone, resulting in cancellous bone tissue. (c) Endochondral ossification type II: Perforating blood vessels providing cells for removal of chondrocytes, with no deposition of bone tissue or cancellous bone formation. Adipose tissue fills the marrow cavity. (d) Intramembranous ossification: Mesenchymal stem cells in collagenous membranes differentiate into osteoblasts, laying down bone tissue and forming an ossification center. The enclosed osteoblasts become osteocytes. Blue strings: Collagen fibers. Created with BioRender.com.

5.2.2 Skeletal muscle

Skeletal muscle in salmonids is arranged in segments, or *myotomes*, visible to the naked eye (fig 6a). The connective tissue separating the myotomes, the *myosepta* (fig. 6b), functions as an attachment for the muscle tissue. The myotomes consist of bundles of myocytes/myofibers and *fascicles*, separated by perimysium, and the myocytes are surrounded by endomysium. One myocyte reaches from one myosepta to the next. Thus, the myosepta functions as a tendon. Mature myocytes have a characteristic position of the numerous nuclei in the periphery of the cell (Fig.6c). The skeletal muscle is classified due to differences in metabolic properties: White skeletal muscle is relatively poorly vascularized, anaerobic and has a low number of mitochondria, responsible for low-enduring and explosive movement. The myocytes are large (50-200 µm) compared to red muscle (Fig 6d) (Fauconneau et al., 1997). Red skeletal muscle, in contrast, is abundantly vascularized, has high amounts of mitochondria, and is aerobic and fatigue-resistant, responsible for long-lasting movement (Kiessling, Ruohonen, & Bjørnevik, 2006). Red muscle cells are smaller in size (25-45 µm) (Fig. 6e). In addition, pink muscle cells representing an intermediate muscle type are found in salmonids (Gunrow, Stange, Bochert, & Tönben, 2021; Kiessling et al., 2006). In contrast to mammals, the different fiber types are not mixed but rather separated in other parts of the truncus,

as illustrated in Figure 4. The white skeletal muscle (*m. lateralis profundus*) makes up over 90 % of the total skeletal muscle mass in salmonids, while the remaining mass constitutes of red muscle fibers (*m. lateralis superficialis*) located laterally on the truncus underneath the skin and pink fibers located between the red and white muscle tissue (Gunrow et al., 2021; Kryvi & Poppe, 2016).

Development and growth of skeletal muscle in salmonid fish is divided into three phases: The embryonic muscle formation, the yolk sac-larval myoblast, and myotome formation, and the following continuous growth throughout life (Johnston, 1999; Stickney, Barresi, & Devoto, 2000; Koumans & Akster, 1995). In contrast to mammals and fish of smaller size, such as zebrafish, the postembryonic growth of the skeletal muscle (myogenesis) in large teleosts is performed through both hyperplasia (increase of myocyte number) that can be observed as relatively small, newly formed cells, and hypertrophy (enlargement of myocytes) seen as large size cells (Rowlerson, Radaelli, Mascarello, & Veggetti, 1997; Kiessling et al., 2006). This is evident in Atlantic salmon, with the number of myotomes increasing from 5,000 at hatching to 1 million in adult-sized fish (Johnston, 1999). In the event of enlargement, the myocytes must recruit additional nuclei provided by adjacent muscle stem cells/satellite cells (Johnston, 1999; Koumans & Akster, 1995). As a lineage of the myogenic progenitor cells, the satellite cells reside in the muscle, located between the basal lamina and plasmalemma of the myofiber (Baghdadi & Tajbakhsh, 2018). These cells can be identified by Pax7, a gene expressed in myogenic satellite cells (Seale et al., 2000). The Pax7 gene has been identified in zebrafish during growth and repair in skeletal muscle (Seger et al., 2011), and satellite-like cells are described in both Atlantic salmon (Matschak & Stickland, 1995) and rainbow trout (Gabillard, Sabin, & Paboeuf, 2010). In mammals and fish, the red muscle cells have a higher nuclei content than equivalent-sized white muscle cells but also a higher number of progenitor cells and satellite cells (Gunrow et al., 2021; Burleigh, 1977). The role of satellite cells during muscle regeneration is vital and is described later in the thesis.

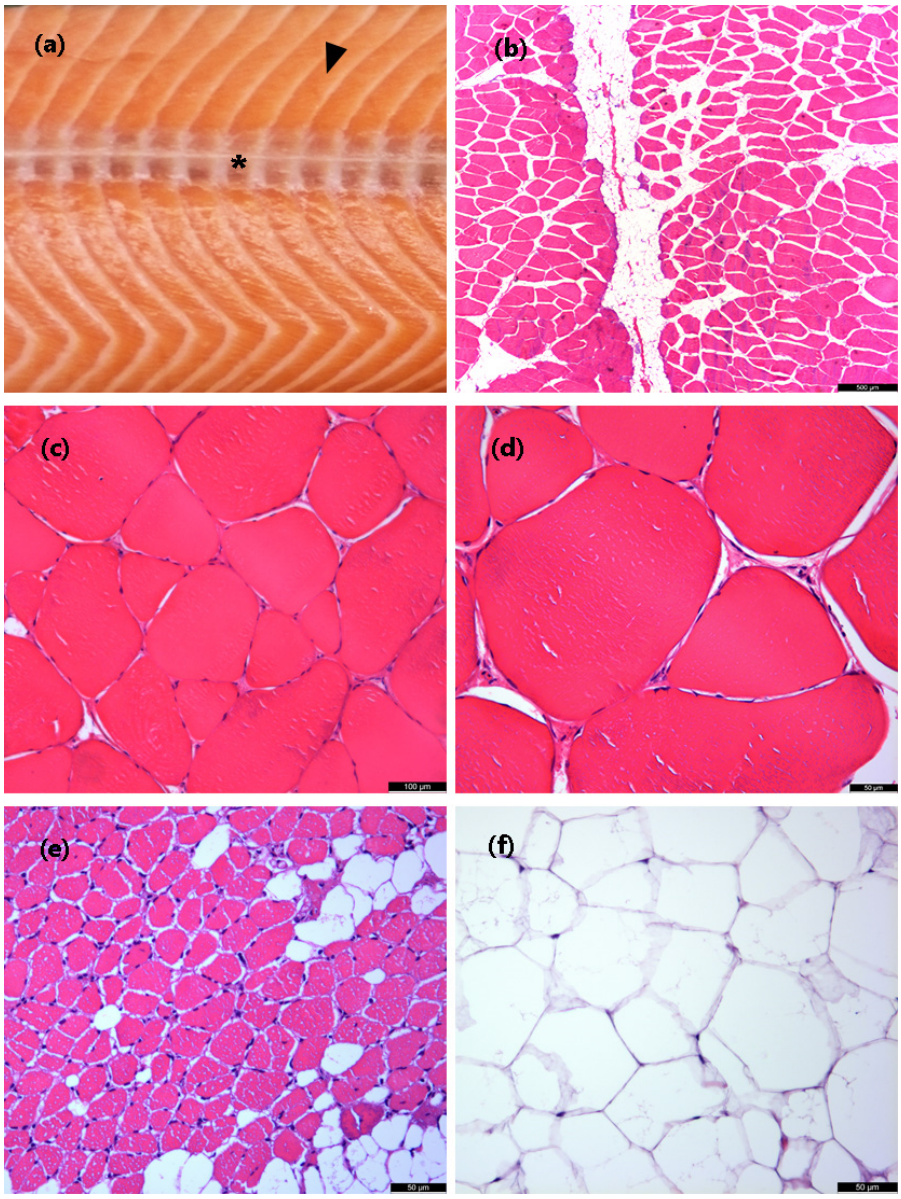

Figure 6. Skeletal muscle in salmonid fish. (a) Macroscopic image of red (asterisk) and white skeletal muscle in Atlantic salmon. Myotomes (arrowhead) are separated by myosepta. (b) Low magnification of skeletal muscle in Atlantic salmon. Myosepta containing adipocytes are separating myotomes. Scale bar= 500 µm.(c) White skeletal muscle in rainbow trout, illustrating a mosaic appearance with different-sized myocytes. Scale bar= 100 µm. (d) Higher magnification of (d), showing the multinucleated myocytes. Scale bar= 50 µm.(e) Red skeletal muscle in Atlantic salmon. The multinucleated myocytes are smaller compared to white skeletal muscle. Scale bar= 50 µm. (f) Adipocytes in rainbow trout. Peripherally located nuclei and a mosaic pattern with different sizes of the cells. Scale bar= 50 µm.

5.2.3 Adipose tissue

In fish, adipose tissue can be divided into visceral/abdominal fat, subcutaneous fat, and so-called consumed fat, the latter representing the adipose tissue found in the skeletal muscle (Weil, Lefèvre, & Bugeon, 2013). Salmonids are classified as fatty fish with abundant lipids in the skeletal muscle. In terms of Atlantic salmon versus rainbow trout, rainbow trout has a higher whole body and fillet crude lipid percentage (Aas, Åsgård, & Ytrestøyl, 2022a) and relatively more excess visceral adipose tissue (Berge et al., 2023). In salmonids, most of the adipose tissue in the skeletal muscle is found in the myosepta in the white skeletal muscle (Fig. 6b) and in the perimysium (Ackman & Zhou, 1994; S. Zhou, Ackman, & Morrison, 1995; Zhou, Ackman, & Morrison, 1996). Adipose cells, or *adipocytes*, vary in size depending on the myoseptal region in the fish (Zhou et al., 1996). Further, lipid droplets can be seen in the endomysium and inside red skeletal muscle myocytes using a light microscope (Ackman & Zhou, 1994; Zhou et al., 1996), whereas lipid droplets in white skeletal myocytes must be visualized using an electron microscope (Nanton et al., 2007). The distribution of lipids coincides with the metabolic properties of the two muscle types: White skeletal muscle utilizes glycogen as the energy source, whereas red muscle performs lipid oxidation through the high number of mitochondria in the myocytes (Nag, 1972).

Adipocytes grow in similar manners as myocytes with hyperplasia and hypertrophy through the differentiation of preadipocytes into adipocytes (Vegusdal, Sundvold, Gjøen, & Ruyter, 2003; Weil et al., 2013). The adipocytes may vary significantly in size (5-200 um) (Fig 6f), and the mean size increases with body weight and lipid content (Fauconneau et al., 1997). The dietary lipid sources can also alter the adipocyte size, seen as enlarged adipose cells in fish fed vegetable oils (Cruz-Garcia et al., 2011; Fauconneau et al., 1997; Bou et al., 2020). In addition to adipocytes, an adipose stromal vascular fraction (aSVF) has been detected in Atlantic salmon adipose tissue by gene expression, representing not only preadipocytes but also vascular cells and lymphocytes, including macrophages (Salmerón, 2018; Todorcević, Skugor, Krasnov, & Ruyter, 2010; Vegusdal et al., 2003). Involvement of the adipose tissue has been confirmed during vaccine-related side effects (Veenstra et al., 2017). Further, mesenchymal stem cells from visceral adipose tissue in Atlantic salmon have been shown to differentiate into mineralizing osteoblast-like cells *in vitro* (Ytteborg et al., 2015). Immune cells have also been reported in the visceral adipose tissue in rainbow trout (Pignatelli et al., 2014).

5.2.4 Growth and measurements in salmonid aquaculture

The fillet is the main product in salmonid aquaculture. Thus, enhanced skeletal muscle growth is an essential trait for selection, and the result of meticulous breeding for a higher growth rate is evident when comparing slaughter weight in farmed versus wild Atlantic salmon kept in common rearing conditions (Glover et al., 2009). As mentioned, teleosts possess indeterminate growth, meaning the skeletal tissues never reach a specific growth plateau (Froehlich et al., 2013; Rescan, 2008). However, the growth rate is influenced by both external and internal factors, such as temperature, maturation, nutritional status, and dietary components (Johnston et al., 2003). The higher growth rate is linked with not only increased skeletal muscle mass but also increased lipid deposition (Todorčević et al., 2009; Todorčević et al., 2008). Increased muscle percentage is the desired outcome, and abundant visceral lipid deposition is a waste product. The ratio of feed intake: weight gain (feed conversion ratio, FCR), i.e., the conversion protein in the feed, is thus also an important parameter used in aquaculture production (Kause, Kiessling, Martin, Houlihan, & Ruohonen, 2016).

Growth and body condition measurements are performed throughout the production to ensure optimal conditions. The growth rate can be reported as absolute or relative (Hopkins, 1992), calculating the weight gain percentage throughout the production. Specific growth rate (SGR) measures the percentage increase in body weight per day. In contrast, the condition factor is calculated using fish length (from the nose to the tail) and body weight, giving a qualitative classification of the fish condition in terms of leanness/fattiness (Barnham & Baxter 1998). The condition factor tends to be higher in rainbow trout than in Atlantic salmon, likely due to differences in weight relative to body length (Berge et al., 2023; Aas et al., 2022a).

Both internal and external factors may influence the growth of aquaculture production. The selective breeding for increased growth rate and slaughter size has resulted in increased hyperplasia of the myocytes and adipose cells, consequently resulting in larger individuals due to hypertrophy of the increased number of these cells (Fauconneau et al., 1997). As increased fat levels in the diet result in increased fat content in the fillet (Einen & Skrede, 1998), research has been conducted to attain reduced visceral lipid weight while ensuring the preferred fillet size to achieve a higher yield of the end product and reduce waste products (Kause, Paananen, Ritola, & Koskinen, 2007). External factors such as nutrient content and feeding regimes highly affect growth (Johansen & Jobling, 1998; Overturf, Barrows, Hardy, Brezas, & Dumas, 2016) and even temperature during egg incubation (Johnston & McLay, 1997; Matschak, Hopcroft, Mason, Crook, & Stickland, 1998), in the freshwater phase (Johnston et al., 2003) and the seawater phase (Jobling, 2002) will affect development and growth through number and size of myocytes and metabolic rate and feed consumption. Additionally, although vaccination is a vital part of salmonid production in Norway, reduced growth can be observed as a side effect post-vaccination (Berg, Rødseth, & Hansen, 2007; Midtlyng & Lillehaug, 1998; Sørum & Damsgard, 2004).

5.3 Inflammation, regeneration and melanization

The immune system is commonly divided into the innate and the adaptive immune system. However, a division between the two is not entirely accurate, as several cellular and humoral (soluble) components can be found in both, and the components herein depend on each other to function. The innate immune system is based on an unspecific first-line defense against pathogens and other potentially harmful agents. In contrast, the adaptive immune system relies on the host's previous encounters with the agent in question, making the defense directed at specific agents. In salmonids, as in other teleosts and mammals, the innate immune system consists of both mechanical barriers such as the skin and scales, mucus, and pH, in addition to the cellular and non-specific humoral components such as macrophages, neutrophils, cytokines, and complement proteins (Plouffe, Hanington, Walsh, Wilson, & Belosevic, 2005). In the adaptive immune system, recognizing specific antigens due to a developed memory creates a fast-acting cellular defense of specific T and B lymphocytes and a humoral defense consisting of antibodies produced by mature B cells.

Even though many of the salmonid immune cells are equivalent to those in mammals, the organization of the immune system based on lymphoid organs and tissues differs greatly. First, there are no apparent lymph nodes, and the existence of lymphatic vessels in fish is disputed (Vogel, 2010). The thymus and the head kidney are primary immune organs, and importantly, as teleosts lack bone marrow, the hematopoietic function is found in the head kidney (Bjørgen & Koppang, 2021). The head kidney also functions as a secondary immune organ, as do the spleen, the

intraepithelial lymphoid tissue of the gills (ILT) and the salmonid bursa (Bjørgen & Koppang, 2021; Haugarvoll, Bjerkås, Nowak, Hordvik, & Koppang, 2008; Løken, Bjørgen, Hordvik, & Koppang, 2020). Additionally, several mucosal-associated lymphoid tissues (MALTs) have been detected in the nose, gut, and gills (Salinas, 2015).

5.3.1 Cells and mediators of the innate and adaptive immune system

The leucocytes in the immunological defense can roughly be divided into granulocytes and agranulocytes (lymphocytes). In mammals, granulocytes are named based on their specific reaction to staining. However, the same is not evident in fish granulocytes (Reite & Evensen, 2006). Basophils are rare in fish and often contain granules of histamine (Dalmo & Bøgwald, 2022), resembling another mammalian granulocyte, mast cells. Mast cells in fish are similar to those in mammals and may be referred to as eosinophilic granule cells (EGCs) (Reite & Evensen, 2006). Neutrophils are indeed present (Havixbeck & Barreda, 2015), but not always to the same extent as in mammalian inflammation responses (Roberts & Rodger 2012). The migration from the circulation is due to molecules such as cytokines and chemokines secreted by cells at the site of damage. Neutrophils are phagocytes and possess the function of cell-mediated cytotoxicity by releasing reactive oxygen species (ROS) and degrading proteases, and neutrophil extracellular traps (NETs) production, thus performing both external elimination and trapping of microbes (Palić, Ostojić, Andreasen, & Roth, 2007). NK cells, or natural killer cells, are lymphocytes with cytotoxic function, recognizing infected or altered cells (damaged or cancerous) and eliminating the cell by inducing cell death. Although specific markers for fish NK cells are not yet developed, there are two analogs to mammalian NK cells found in fish: The non-specific cytotoxic cell (NCC) and the NK-like cell (Dalmo, Ingebrigtsen, & Bøgwald, 1997; Fischer, Koppang, & Nakanishi, 2013; Nakanishi, Toda, Shibasaki, & Somamoto, 2011). In fish, red blood cells and thrombocytes also possess immunological properties (Köllner, Fischer, Rombout, Taverne-Thiele, & Hansen, 2004; Ortega-Villaizan, Coll, & Rimstad, 2022).

Professional antigen-presenting cells (APCs), such as macrophages and dendritic cells, are essential links between the innate and adaptive immune response. Dendritic cells with similar functions and phenotype as seen in mammals have been characterized in zebrafish (Shao et al., 2015)**.** Macrophages are agranulocytic cells arriving at the site of injury or infection shortly after neutrophils (Crane & Albina, 2014). These cells are phagocytic and will take up harmful agents and debris from dead cells. In mammals and fish, macrophages can be categorized based on their polarization: M1 macrophages produce pro-inflammatory cytokines and nitric oxide (NO), or M2 macrophages produce anti-inflammatory cytokines and arginase (Galdiero, Biswas, & Mantovani, 2014; Wentzel et al., 2020a; Wentzel et al., 2020b). In mammals, a distinction is made between recruited bone marrow-derived macrophages and M2-polarized tissue-resident macrophages originating from the embryonic yolk sac (Davies, Jenkins, Allen, & Taylor, 2013; Ito, Hikosaka-Kuniishi, Yamazaki, & Yamane, 2022). The distinction between recruited and tissue-resident macrophages can be made by specific antibodies (ED1 vs. ED2/ED3, respectively), as shown in rats (Dijkstra, Döpp, Joling, & Kraal 1985). Tissue-resident macrophages, such as Langerhans cells, have been detected in fish (He et al., 2018), in which the majority originates from hematopoietic stem cells. However, some tissue-resident macrophages in the zebrafish epidermis are of non-hematopoietic origin (Lin, Wen, & Xu, 2019). Macrophages in mammals and fish show many similarities (Dalmo & Bøgwald, 2022). Still, an important distinguishment from higher vertebrates is the presence of melanin produced by macrophages known as melano-macrophages (Haugarvoll, Thorsen, Laane, Huang, & Koppang,

2006). These cells are of particular importance to this thesis and will be described in detail in later sections.

Altogether, the cells involved in an immunological response aim to eliminate the damaging agent, remove dead or damaged cells and tissue, and restore the tissue function and structure. However, the cells cannot function without a signaling system to induce the reactions. Mediators such as cytokines, chemokines, and receptors are vital to performing a cellular defense. When a cell or tissue is damaged and/or infected, damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) are present. These molecules are displayed on injured and dying host cells or derived from pathogenic agents, respectively, such as bacterial lipopolysaccharides and viral nucleotides (Magnadottir, 2006). To discover the presence of DAMPs and PAMPs, receptors recognizing these patterns are vital. Pattern-recognizing receptors (PRRs) are non-specific receptors enabling the innate immune defense to conduct inflammatory reactions without a previous pathogen encounter. Numerous cell types express these receptors in the body, and activation of PRRs will induce a signaling cascade, resulting in the upregulation of immune-related genes. Depending on the pathogen in question, i.e., extracellular or intracellular species and strains, different PRRs may be activated: Several toll-like receptors (TLR), retinoic acid-inducible gene I (RIG-I)-like receptors and nucleotide oligomerization domain (NOD)-like receptors are all present in fish (Dalmo & Bøgwald, 2022). As the PRRs recognize DAMPs or PAMPs, the cell will go into an anti-pathogen state, with the production and release of several cytokines and mediators such as interferons (IFN type I and II), tumor necrosis factor α (TNFα), complement proteins, and antimicrobial peptides (Sun, Robertsen, Wang, & Liu, 2009) with many functions, such as stimulating antigen presentation, opsonization of agents, inhibition of viral transcription and controlling apoptosis (Hanada & Yoshimura, 2002).

The cells of the adaptive immune system in fish comprise T-lymphocytes and B-lymphocytes (T-cells and B-cells), as in mammals. Several characteristics can be found similar to that of higher vertebrates, such as cytotoxic T-cells, CD4/CD8-receptors, CD3 co-receptors (Fischer et al., 2013), and major histocompatibility complex (MHC)-molecules. The production of specific piscine immunoglobulins (IgM, IgT, IgD) by B-cells (Hikima, Jung, & Aoki, 2011) makes it possible to conduct several functional and structural investigations on the immune system of teleosts. To activate the cells of the adaptive immune system, antigens must be presented by infected or phagocytic cells by MHC class I or II complexes (Mantegazza, Magalhaes, Amigorena, & Marks, 2013). However, B-cells in fish are highly phagocytic, thus functioning as a part of the innate immune system (Zhang et al., 2017).

5.3.2 Melano-macrophages and melanin

In mammals and birds, cells containing melanin pigment are restricted to include classical melanocytes derived from the ectoderm in the skin and non-classical melanocytes in internal organs, such as neuroepithelial cells of the retina. Non-classical melanocytes are defined by their localization in tissues other than skin and/or with an alternative migratory pathway to the neural crest-derived classical melanocytes (Colombo, Berli, Delmas, & Larue, 2011; Arnheiter & Debbache, 2021). Melanophages, conversely, are phagocytic cells in the skin internalizing melanin, particularly during pathological conditions (Weiss, James, & Cooper, 1988). Melanin is synthesized in specialized organelles in the melanocytes named melanosomes. In the epidermis, melanosomes are secreted by classical melanocytes, followed by an internalization by keratinocytes, while dermal melanocytes

interact with fibroblasts, without the transfer of melanosomes (Colombo et al., 2011; Moreiras et al., 2022). In lower vertebrates such as fish, melanophores are equivalent cells to melanocytes. (Colombo et al., 2011). There is no evidence of melanin-synthesizing macrophages in higher vertebrates. However, such phagocytes can be found in reptiles, amphibians, and fish (Haugarvoll et al., 2006; Sichel, Scalia, Mondio, & Corsaro, 1997).

Melanin-containing macrophages, commonly known as melano-macrophages, are thought to originate from myeloid cell lines in the hematopoietic tissue in fish. Their appearance occurs after the first feeding, coinciding with immunological maturity, in immune organs such as the head kidney, spleen, and liver (Agius, 1981). The abundance and distribution of melano-macrophages in fish varies among species (Meseguer, Lopez-Ruiz, & Esteban, 1994; Roberts, 1975) but also depends on age (Agius, 1981), inflammatory status, and environmental differences and challenges (Agius & Roberts, 2003). Aggregates of melano-macrophages, known as melano-macrophage centers (MMCs), are found in both fish, amphibians, and reptiles (Ahmed, 2022; Steinel & Bolnick, 2017; Wu, Zhang, Chai, & Wang, 2017). In advanced teleost species, MMCs are commonly seen in the head kidney and spleen (Agius & Roberts, 2003; Wolke, 1992), while in primitive teleosts such as salmonids, melano-macrophages are less organized and more randomly distributed in the interstitium of these organs, often located around vessels (Roberts, 1975; Schwindt et al., 2006). In salmonids, the degree of pigmentation, i.e., the amount of melanin in the melano-macrophages, are abundant compared to many other teleosts species (Agius, 1985). Melano-macrophages have been shown to retain antigens for a prolonged period, indicating that these cells perform long-lasting presentation of antigens to cells of the adaptive immune system (Brattgjerd & Evensen, 1996; Herraez & Zapata, 1986). Thus, due to their functional and structural similarities and the implication that the organization of the cells follows vertebrate evolution, MMCs are suggested to be analogs to germinal centers (GC) in higher vertebrates (Agius, 1980). However, some essential features in mammalian germinal centers have not yet been detected in fish MMCs (Agius, 1985; Press & Evensen, 1999; Steinel & Bolnick, 2017). Nevertheless, melanin-containing cells have important properties in terms of cellular protection of the organism.

Melanin is a heterogeneous group of pigments best known for contributing to the defense against UV radiation in the skin of mammals (Riley, 1997; Wolke, 1992). This is true, but the pigment is seen in numerous biological systems, including plants, bacteria, insects, amphibians, and fish. The function of melanin has light absorbance, antioxidant functions (redox properties), and binding of metal ions, and may also bring different modulations of the immune system, such as suppression of proinflammatory cytokine production (Christensen, Li, Chen, & Nappi, 2005; ElObeid, Kamal-Eldin, Abdelhalim, & Haseeb, 2017; Mohagheghpour et al., 2000; Riley, 1997). In mammals and fish, melanin production is performed through a series of enzymatic steps, starting with the amino acid tyrosine. The end products are eumelanin or pheomelanin, and the two are distinguishable due to their synthesis pathway, solubility, and color: Brown/black and insoluble and red/brown and soluble, respectively. The synthesis of eumelanin involves key enzymes coded by the tyrosinase gene family, herein tyrosine-related protein 1 (Tyrp-1) and tyrosine-related protein 2 (Tyrp-2) (Boonanuntanasarn, Yoshizaki, Iwai, & Takeuchi, 2004; Wakamatsu & Ito, 2021). In this thesis, the term melanin is used for eumelanin, which is visible as dark intracellular pigments on hematoxylin and eosin staining. The presence of pheomelanin in fish is disputed; however, with the use of different histological staining methods, pheomelanin is suggested to be present in at least some teleost species (Dang et al., 2019; Kottler, Künstner, & Schartl, 2015). In addition to melanin produced by the cell, other pigments may be found in melano-macrophages. Lipofuscin, a yellow/brown pigment resulting from the oxidative polymerization of polyunsaturated fatty acids, may accumulate during bacterial or viral infections or due to poor nutrition (Agius, 1985; Roberts &

Rodger, 2012). Hemosiderin, an iron-containing pigment from the catabolism of hemoglobin, is primarily seen in melano-macrophages associated with MMCs in the spleen (Agius & Roberts, 2003)

For many years, the melanin seen in melano-macrophages of fish was thought to result from phagocytic activity (Agius, 1985), as seen in melanophages in mammals. However, in 2006, synthesis of melanin in macrophages in Atlantic salmon was discovered in vitro in the salmon head kidney (SHK)-1 cell line, showing the production and secretion of melanosomes through tyrosinase-activity in a teleost leucocyte cell line (Haugarvoll et al., 2006). These cells were CD83-positive, a marker for dendritic cells. Recently, tyrosinase activity was also described in a CD83-negative cell line from tilapia head kidney (THK) with macrophage characteristics, indicating these cells to be melano-macrophage progenitors (Wen, 2016). Melanogenesis has also been shown to occur in organized granulomas in pigmented skeletal muscle in Atlantic salmon (Larsen et al., 2012) and melanized blood vessels in cod (Cooper & Midling, 2007). Muscle melanization has been observed in fish with elevated zinc levels in the diet of sand flathead *(Platycephalus bassensis)* (Ooi et al., 2022). Thus, the presence of melano-macrophages and melanin in non-immunological organs and tissues has been described most commonly in fish suffering from infections and internal lesions (Dang et al., 2019; Koppang et al., 2005; Larsen et al., 2012; Overstreet & Thulin, 1989). In healthy fish, however, the distribution of melano-macrophages in non-immunological and non-mucosal organs is less described but has been observed in the heart, for example (Overstreet & Thulin, 1989). Generally, the presence of tissue-resident macrophages in fish's skeletal muscle and adipose tissue is not well described but has been reported in mammals (Hassnain Waqas et al., 2017; Wang et al., 2020). This will be further addressed later in the thesis.

5.3.3 External and internal factors affecting the immune response

Fish are in close and constant contact with the external environment and depend on a robust but balanced innate immune response. Both external and internal factors can influence the immune response. External factors include water temperature, water quality, nutrition, and infectious agents in the milieu. In poikilothermic organisms such as salmonids, the body temperature adapts to the surrounding environment, and thus, the metabolic reactions will be influenced equally. This will affect the inflammatory response rate (Fryer et al., 1976), although non-specific immune responses can provide compensatory actions during low water temperatures (Bly & Clem, 1992; Le Morvan, Troutaud, & Deschaux, 1998). In any organism, adequate nutrition is vital for survival, and particularly in aquaculture, nutrition is of great importance as malnutrition can impact fish growth and, consequently, production. Minerals, vitamins, proteins, and lipids in fish feeds have proven to play vital roles in immune responses in fish (Jantawongsri, Jones, Elliot, Scmidt-Posthaus, & Nowak, 2022), including salmonids (Kiron, Fukuda, Takeuchi, & Watanabe, 1995). Regarding lipid composition, the nutritional content of long chain-polyunsaturated fatty acids (LC-PUFA) has proven to be of great importance to commercial farming productions. LC-PUFAs are divided into two groups, n-6 and n-3 PUFAs (Venegas-Calerón, Sayanova, & Napier, 2010). Due to cost efficiencies, the dietary lipid level in commercial salmonid feeds consists of a high proportion of vegetable oils (VO), replacing the preferred fish oils (FO) (Aas, Åsgård, & Ytrestøyl, 2022b). This leads to a higher level of pro-inflammatory n-6 fatty acids (Montero et al., 2010) and a lower level of the anti-inflammatory n-3 highly unsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), resulting in a negative impact on fish health and robustness (Bou et al., 2017). Thus, influenced by

both the temperature and nutritional status, the pathological changes inflicted by harmful agents vary in severity and duration.

Vaccination and stress must be included in aquaculture production when considering possible external factors influencing the immune response. Vaccination is important in preparing the immune system and preventing disease (Midtlyng 2022). In Norwegian aquaculture, vaccines against bacterial infections such as classical furunculosis (Midtlyng, Reitan, & Speilberg, 1996) have proven very successful, dramatically reducing antimicrobial treatment use (Norwegian Seafood Council, 2021). Vaccines against viruses are more challenging to develop, much due to their intracellular viral replicating mechanisms. However, some vaccines are successfully used against specific viral infections (Ma, Bruce, Jones, & Cain, 2019). Although with a successful outcome regarding protection against many diseases, vaccination brings side effects, both local and systemic (Midtlyng & Lillehaug, 1998). During intraperitoneal injections, adjuvants will induce a local inflammation in the abdominal cavity, resulting in possible adhesions and chronic inflammation in the local tissue (Poppe & Breck, 1997). Moreover, the handling procedure during vaccination is stressful for the fish. Stress induces a cascade of processes in the organism, including nervous, endocrine, and immunological responses (Tort & Balasch, 2022). As in mammals, cortisol is a vital stress hormone in fish, and during both acute and chronic stress, cortisol concentrations are elevated. The number of immune cells is correspondingly affected (Grzelak et al., 2017). The result of these mechanisms is evident in farmed salmonids, as disease outbreaks may occur after stressful periods (Taksdal, Ramstad, Stangeland, & Dannevig, 1998). Noteworthy, several production-related factors in aquaculture have shown to influence MMCs, such as nutrition and stocking density (Montero, Blazer, Socorro, Izquierdo, & Tort, 1999).

In terms of internal or genetic factors influencing the immune response, the diverse variations found in both susceptibility and response to disease in different vertebrate species are evident. Although, as mentioned, properties of the immune system are highly conserved throughout evolution, the response to trauma and intruding agents may differ between species, as seen during intraperitoneal injection of adjuvanted vaccines in Atlantic salmon and rainbow trout (Mutoloki, Reite, Brudeseth, Tverdal, & Evensen, 2006), but also within the same species due to both external and genetic divergencies. The genes code for factors important in both innate and adaptive immunity (Buchmann, 2022); thus, as breeding in aquaculture has evolved to become highly advanced, including genomic selection (GS) and marker-assisted selection (MAS), specific traits providing higher protection against diseases has been selected for. In salmonid production, several genetic markers have been identified as necessary in relation to resistance to infection and disease, such as quantitative trait loci (QLT) associated with resistance against infectious pancreatic necrosis virus (IPNV) (Houston et al., 2008; Moen, Baranski, Sonesson, & Kjøglum, 2009) and salmonid alphavirus (SAV) in Atlantic salmon (Aslam et al., 2020). Here, both early onset of the adaptive defense and lower early viral replication have been reported to improve resistance (Purcell, LaPatra, Woodson, Kurath, & Winton, 2010). The genetic divergencies between species or within separate groups of the same species can thus result in different susceptibility and responses to an infection.

5.3.4 Inflammatory reactions in the musculoskeletal system

In teleosts, the acute inflammation is similar to that of mammals, as equivalent cells and mediators are present in the inflammatory response. Despite the many common features, as mentioned, some major differences are bound to influence the fish's immune response: Their anatomical divergencies, their life in an aquatic milieu, and their poikilothermic properties. Nevertheless, in the event of tissue damage and necrosis caused by infections, mechanical trauma, hypoxia, and other harming factors leading to acute inflammation, the immune system will respond in the way most fitting for the factor in question. The acute inflammatory response in mammals is induced by mediators produced by cells at the site of damage (Martin & Feng, 2009). First, during the *vascular phase*, an increase in blood flow and permeability in the vessels will allow the passage of proteins, plasma, and migration of neutrophils followed by macrophages and other leucocytes, known as the *cellular phase* (Ackermann, 2012; Loynes et al., 2010). By their function, neutrophils, NCC, and NK-like cells are important for eliminating microbes but will, in turn, create a pro-inflammatory milieu due to the tissue damage following the secretion of degrading components. The initial tissue damage combined with the inflammatory milieu will attract additional immune cells to the site. The leukocyte migration is possible through chemoattractants released by affected host cells and intruding agents and the polymerization of the plasma protein fibrinogen (Manseth et al., 2004). Thus, at the damage site, inflammation is promoted by local and migrated cells and mediators, enabling the removal of the harmful agent but simultaneously inflicting damage to the local tissue. The *reparative phase* of acute inflammation consists of removing necrotic cells and debris, along with mesenchymal stem cell differentiation, to restore the initial function and structure of the tissue. In severe tissue damage, connective tissue replaces areas where normal structure and function are not restored, leaving a scar formation (Ackermann, 2012).

In mammals and fish, several possible outcomes can occur following an acute inflammatory response, depending on the efficiency of the reaction. Should the harmful agent be efficiently removed and the area of affected tissue is limited, healing and regeneration will restore tissue structure and function (Soliman & Barreda, 2022). Should the affected area include more extensive areas and necrosis and loss of parenchymal cells are abundant, fibrovascular tissue and later loose connective tissue will fill the space resulting from the removal of necrotic debris. Collagen-rich connective tissue will further replace the loose connective tissue, restoring the structure but not the function of the tissue. A chronic inflammatory response will develop if the harmful agent persists in the tissue, as in some intracellular infections or foreign body reactions (Shah, Pritt, & Alexander, 2017). On a structural level, chronic inflammation is characterized by the causative factor and the immune response against it. Thus, the distribution may be focal, multifocal, or diffuse. When macrophages dominate a chronic inflammation, the changes are usually described as *granulomatous inflammation* (Williams & Williams, 1983). In fish, macrophages may develop into epithelioid cells and multinucleated giant cells (MGCs) (Noga, Dykstra, & Wright, 1989; Roberts & Rodger, 2012) as seen in mammals, and may be randomly distributed or located in relation to organized granulomas, thus giving the granulomatous inflammation a diffuse or nodular appearance, respectively (Shah et al., 2017).

The zebrafish model is well-used for investigating similarities and possible differences in developmental, pathological, and immunological conditions in fish and mammals. Zebrafish (*Danio rerio*) is a primitive teleost species in the family *Cyprinidae* and has become a widely used laboratory fish in medical research due to the great genetic similarities to mammals, including humans,

combined with fast development and low-cost demands (Briggs, 2002). Thus, thorough work has been conducted on the inflammatory response in fish versus mammals over the last decades (Campos-Sánchez & Esteban, 2021). It is, however, important to remember the great diversity of the 28,000 different teleost species, and the inflammatory and regenerative capacity will thus depend on the species and, importantly, on the organ or tissue in question. Therefore, inflammation and regeneration in the tissues of the musculoskeletal system of farmed Atlantic salmon and rainbow trout are given special attention in this thesis.

5.3.4.1 Pathological conditions and regeneration in bone tissue

In salmonid aquaculture, deformities of the axial skeleton of Atlantic salmon and rainbow trout affecting the vertebral column have been given much attention due to both lower fish welfare and performance, and consequently, a negative impact on the production yield (Deschamps et al., 2008; Witten, Gil-Martens, Huysseune, Takle, & Hjelde, 2009). Many factors in the aquaculture industry can affect vertebral properties and morphology (Fjelldal et al., 2012), such as fast growth through rearing procedures (Fjelldal et al., 2006), nutrition (Fjelldal et al., 2009), vaccination (Berg, Yurtseva, Hansen, Lajus, & Fjelldal, 2012) and exercise levels (Deschamps et al., 2009; Totland et al., 2011). Genetic divergencies between farmed and wild Atlantic salmon has little impact on the presence of vertebral deformities (Fjelldal, Glover, Skaala, Imsland, & Hansen, 2009); however, as shown by Gislason (2010), genetic mutations affecting bone development can be the cause of some skeletal deformities, including abnormal ribs, seen in rainbow trout (Gislason et al., 2010). It is likely that factors affecting the bone tissue development and structure in the vertebral column also affect other bone tissue structures in the fish. For example, costal deformities have been detected together with vertebral deformities in fish with phosphorus deficiency (Baeverfjord et al., 1998). Costal deformities are not given as much attention as other parts of the axial skeleton, likely due to their lower impact on production. The prevalence of rib deformities, including costal fractures in farmed Atlantic salmon and rainbow trout, has been given some attention (Boglione et al., 2014; Jiménez-Guerrero et al., 2022), but has not been investigated to a large extent.

A life spent in an aquatic environment follows an altered impact from gravity compared to terrestrial vertebrates. This results in a larger mass of skeletal muscle in relation to the axial skeleton bone tissue, unlike in mammals (Stickney et al., 2000). As mentioned, although relatively thin and flexible, teleost ribs show a high breaking force (Fiedler et al., 2021), likely to withstand the constant strain of the attached skeletal muscle during continual movement. This is reflected by the higher mineral content in the bones of fish exposed to a higher activity level (Totland et al., 2011). Thus, normal swimming movement is not expected to result in costal fractures. However, costal fractures have been reported in both wild and farmed fish (Fjelldal et al., 2020; Fjelldal et al., 2018). This leads to the question of whether external trauma or other pathological conditions in the bone may cause the ribs to fracture. In mammals, several osteopathological conditions will affect and weaken the strength of the bone, such as osteoporosis (OP); reduced bone mineral density (Christodoulou & Cooper, 2003), osteogenesis imperfecta (OI); altered bone structure and composition (Nijhuis et al., 2019), and osteomalacia (OM); defect bone mineralization (Maricic, 2008). Both OP and OI have been observed in zebrafish during experimental investigations (Fisher, Jagadeeswaran, & Halpern, 2003; Rosa, Laizé, Gavaia, & Cancela, 2021), while OM is an observed consequence of phosphorus deficiency in fish, including salmonids (Baeverfjord et al., 1998; Baeverfjord et al., 2019; Witten et al., 2019). Therefore, the phosphorous level in commercial fish feed is adjusted to meet the need for normal bone development. In terms of nutrition and the hot topic of vegetable oils in replacement

for fish oils, it is reported that although lower mineralization is observed during smoltification in fish fed vegetable oil-based feeds, there is no difference in skeletal deformities in the two feeds in adult fish (Berge et al., 2009). All in all, the costal fractures in fish could be due to pathophysiological conditions or external trauma causing breakage. The latter might be more likely to occur in aquaculture production as the fish is handled during transportation, vaccination, and medical treatment. However, knowledge is still scarce on these assumptions.

As for fracture healing, many reports have investigated detailed bone tissue repair in several teleost species, both in cellular and acellular bone (Moss, 1962). The reports conclude that this process is similar to that of mammals, containing both an initial phase with hemorrhage due to disturbance of the local vascularization (de Haan, Fosseidengen, Fjelldal, Burggraaf, & Rijnsdorp, 2016), quickly followed by the recruitment of osteoblasts and osteoclast to perform callus formations and subsequently remodeling of the newly laid down (woven) bone best to restore the original structure and function of the bone (Fjelldal et al., 2018; Takeyama et al., 2014; Tomecka, Ethiraj, Sánchez, Roehl, & Carney, 2019).

5.3.4.2 Inflammation and regeneration in skeletal muscle

The regeneration response in skeletal muscle is a highly conserved process through evolution. Although the processes may differ greatly among species, some mechanisms are conserved: The ability for the myocytes to re-enter the cell cycle by dedifferentiation into mononuclear and proliferative cells and the contribution of satellite/satellite-like cells (Baghdadi & Tajbakhsh, 2018). Dedifferentiation is mainly seen in species with limb-regenerative capacities (Sandoval-Guzman et al., 2014), and in fish, dedifferentiation of myocytes following tissue injury is seen in extraocular muscles (Saera-Vila et al., 2015). Dedifferentiation has largely been lost in vertebrates, but the presence and importance of satellite cells, on the other hand, is crucial in lower and higher vertebrates (Siegel, Gurevich, & Currie, 2013). As mentioned, during both pre- and postembryonic growth, satellite cells are recruited for hyperplastic growth, regeneration, and development of restored muscle in the event of muscle injury.

The regeneration process in muscle can be divided into three phases, which can also be detected by histological examinations: The *proliferating phase* of satellite cells, including necrosis of injured myocytes and a subsequent inflammation; the *early differential phase* of satellite cells into myoblasts, with newly formed myocytes with central nuclei, including removal of cell debris by immune cells and the presence of loose connective tissue, and the *late differential phase,* where hyperplasia and hypertrophy of myocytes are observed, including more organized dense connective tissue and newly formed vessels (Baghdadi & Tajbakhsh, 2018; Tidball & Villata, 2010; Winkler et al., 2011). As the myocytes are harmed, either by physical trauma, physiological disturbances, or invading pathogens, intracellular components will leak through the compromised sarcolemma, which will activate the quiescent satellite cells (Fu, Wang, & Hu, 2015) to enter the cell cycle (proliferation phase). This attracts a fast influx of immune cells, first neutrophils and macrophages and later T- and B-cells, which will further facilitate the proliferation and differentiation into myoblasts (early differential stage) and fuse to form new myocytes (late differential stage) (Yang & Hu, 2018). As mentioned, essential properties, the poikilothermic properties, are an important factor influencing the regeneration time course in fish. Thus, all the stages mentioned above occur in fish, as seen both as upregulation of immune gene expression in Atlantic salmon myocytes (Pooley, Tacchi, Secombes, & Martin, 2013) and in fish skeletal muscle during bacterial infection (Valenzuela

et al., 2017), however with some variance in time of onset and duration. Mosaic patterns in the skeletal muscle of larger species are seen in intact muscle and regenerating muscle with smaller, newly formed, regenerating muscle cells adjacent to larger muscle cells at the later stages of regeneration (Rowlerson et al., 1997). In zebrafish, the myosepta have been suggested as a contributor during inflammation, either as a route during the migration or as a source for regenerating fibers. However, this is not seen in larger fish with post-larval continuous growth (Rowlerson et al., 1997).

The immune response will affect the regeneration process of skeletal muscle, as proinflammatory cells and molecules will promote the proliferation stage, and anti-inflammatory cells and molecules will enhance the differentiation stage of myogenesis (Ziemkiewicz, Hilliard, Pullen, & Garg, 2021). Macrophages are of specific importance, implicated by the role of the pro-inflammatory and anti-inflammatory phenotypes on proliferation and differentiation of satellite cells during the different regeneration stages (Arnold et al., 2007; Massimino et al., 1997; Merly, Lescaudron, Rouaud, Crossin, & Gardahaut, 1999), and through the general healing and fibrosis in injured skeletal muscle (Muñoz-Cánoves & Serrano, 2015; Tidball, 2010). Further, subpopulations in M2 polarized macrophages affect healing and regeneration through different mechanisms (Villalta, Nguyen, Deng, Gotoh, & Tidball, 2009; Ziemkiewicz et al., 2021). The polarization of M1 and M2 can be affected by the phagocytic activity of the macrophages (Arnold et al., 2007), mechanical strain (Ballotta, Driessen-Mol, Bouten, & Baaijens, 2014), and iron overload in the tissue (Sindrilaru et al., 2011), among others, and is still a source of research in mammalian medicine today. An imbalance in the strict regulation of M1 and M2 presence in the tissue is an important factor in chronic inflammatory conditions in the skeletal muscle in mammals, such as Duchenne muscular dystrophy (DMD) (Dort, Fabre, Molina, & Dumont, 2019). Hybrid phenotypes (a hybrid of M1 or M2) has been detected in traumatic injured skeletal muscle in mouse seen with both regenerative and fibrotic tissue, indicating the cause of damage to affect the outcome of healing by affecting the phenotype polarization (Novak, Weinheimer-Haus, & Koh, 2014).

In mammals, although without phagocytic activity and generally viewed as M2-phenotypes, the tissue-resident macrophages play important roles in the total macrophage response in skeletal muscle (Davies et al., 2013; McLennan, 1993). These cells are located in the perimysium and epimysium of the muscle and are important during the recruitment of inflammatory cells from the circulation (Brigitte et al., 2010). Apart from these tissue-resident macrophages, extensive research in later years has shown important contributions by other cells already present in the skeletal muscle interstitium, such as fibro-adipogenic progenitor cells (FAPs). FAPs are stem cells that may differentiate into fibroblasts or adipocytes, and during acute skeletal muscle injury, FAPs rapidly increase and positively affect myogenesis (Joe et al., 2010). The different macrophage phenotypes regulate FAPs, as proinflammatory M1 will induce apoptosis of FAPS and thus reduce the extent of adipose tissue and fibrosis deposits. In contrast, M2 will induce FAP differentiation (Lemos et al., 2015). Although FAPs may promote satellite cell differentiation in acute injury, the prolonged inflammation during chronic myopathies may lose this effect, and the fibrotic scar tissue will be a dominant feature (Mozzetta et al., 2013). Thus, due to the unbalanced inflammatory milieu, FAPs give rise to both extensive ectopic adipose tissue (Uezumi, Fukada, Yamamoto, Takeda, & Rsuchida, 2010) and fibrosis (Uezumi et al., 2011) during failed regeneration of skeletal muscle. Other cells resident in the skeletal muscle that contribute to the inflammation and regeneration during injury are endothelial cells and pericytes, both of which are involved in angiogenesis, revascularization, and satellite cell regulation (Birbrair et al., 2014; Christov et al., 2007).

The cause of injury will also affect the regeneration response, as illustrated in Atlantic salmon and rainbow trout during infection with the gram-negative bacteria, activating PAMPs, versus mechanical trauma, activating DAMPs (Ingerslev, Lunder, & Nielsen, 2010). Here, the infection shows a higher expression of proinflammatory cytokines. Similarly, if the pathogen is extracellular or intracellular, the response will happen accordingly. For example, a common response to viral infections is autophagy of the infected cell (Cui, 2019), a mechanism seen in close relation to the immune response in rainbow trout skeletal muscle cells (Valenzuela et al., 2023). However, the immune response to pathogen infection depends highly on the specific pathogen in question and its mechanisms to invade the cell and avoid the host defense system.

5.3.4.3 Inflammatory conditions in adipose tissue

Inflammation of adipose tissue, also known as steatitis, has been described in several teleosts species, particularly farmed fish. Pansteatitis is characterized as a generalized inflammatory process in the adipose tissues and has been associated with certain types of nutritional fish oils combined with low levels of vitamin E. The increased amount of LC-PUFA in the feed will increase the demand for vitamin E due to increased oxidation activity (Farwer et al., 1994). In earlier reports on pansteatitis in farmed fish, the affected fish were recognized by abnormal and lethargic swimming behavior and a darker color fin (Roberts & Agius, 2008; Roberts, Richards, & Bullock, 1979). Here, histological examinations revealed that several organs were affected, with infiltration of lymphocytes and macrophages. Skeletal muscle was also compromised, suggested to result from a low vitamin E level in the diet. (Roberts et al., 1979). However, steatitis can be detected in fish without macroscopic or behavioral alterations. In rainbow trout, the condition was confirmed through the detection of granulomas in the adipose tissue in fish fed high fish oil (FO) based diet (Twibell et al., 2017), suggesting this condition to be more prevalent but not easily discovered and that a reduction in FO in the diet could be beneficial.

On the contrary, the benefits of dietary FOs are widely accepted in obesity treatment in mammals (Bargut, Mandarim-de-Lacerda, & Aguila, 2015). Steatitis is commonly linked to obesity in mammals suffering from metabolic syndrome (Hotamisligil et al., 2006; O'Rourke, 2009). The enlarged adipocytes release proinflammatory molecules named adipokines (Fasshauer & Blüher, 2015; Skurk, Alberti-Huber, Herder, & Hauner, 2007) during obesity, causing insulin resistance (Hotamisligil, Murray, Choy, & Spiegelman, 1994; Xu et al., 2003). The inflammatory response is suggested to be induced by oxidative stress or hypoxia in the adipose tissue (Pasarica et al., 2009; Trayhurn, Wang, & Wood, 2008). In obese individuals, adipose tissue is characterized by enlarged adipocytes and infiltration of inflammatory cells, herein an abundance of M1-polarized macrophages (Schenk, Saberi, & Olefski, 2008; Suganami & Ogawa, 2010). The increased amount of bone marrow-recruited macrophages relative to the M2-polarized tissue-resident macrophages (Bourlier & Bouloumie, 2009) correlates with increasing adipocyte size (Weisberg et al., 2003) and is seen in close relation to dying adipocytes (Saverio et al., 2005). These mammal studies show that adipose tissue can greatly recruit the immune system during compromised metabolism and local and systemic disturbances.

As mentioned in earlier sections, adipose tissue makes up a significant part of the total body mass in fish. The inflammatory conditions in obese mammals have led to an increasing interest in studying similar changes in farmed salmonids, as deposition of excess visceral fat is a consequence of increased lipid levels in the feed, resulting from hyperplasia and, particularly in larger individuals, hypertrophy of adipocytes (Gélineau, Corraze, Boujard, Larroquet, & Kaushik, 2001). The reduced

level of level of EPA and DHA in commercial fish feeds is shown to affect the deposition of visceral adipose tissue (Bou et al., 2017; Todorčević et al., 2009) and the adipocytes are also likely to increase in size due to increased storage of lipids on a VO diet (Todorčević et al., 2008). Increased lipid storage and low levels of DHA are likely linked to elevated oxidative stress related to increased inducible nitric oxide synthase (iNOS) and impaired mitochondrial activity (Bou et al., 2020; Todorčević et al., 2009). Regarding species differences, recent studies have shown that rainbow trout has a better capacity to increase EPA and DHA metabolism (Berge et al., 2023).

5.3.4.4 Selected viruses affecting the salmonid musculoskeletal system

Several infectious agents can cause disease during the production cycle of Atlantic salmon and rainbow trout. Disease outbreaks can cause high morbidity and mortality and are therefore a threat to both the production outcome and animal welfare. As mentioned, vaccines have successfully encountered many diseases caused by bacterial infections. However, vaccines protecting against diseases caused by viruses are more complicated to obtain. In relation to the topic of this thesis, some viral infections are of particular interest as they affect the skeletal muscle and are therefore relevant when addressing inflammation in the musculoskeletal system. Thus, a specific focus is given in the next section on these viruses.

Piscine orthoreovirus-1 (PRV-1)

Piscine orthoreovirus 1 is the causative agent of the disease known as heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (Palacios et al., 2010; Wessel et al., 2017). The non-enveloped, double-stranded RNA virus belongs to the family *Reoviridae* (Markussen et al., 2013) and is ubiquitous in the seawater phase of salmonid production (Løvoll et al., 2012). The virus has also been detected in wild Atlantic salmon, but infection of the virus is not consistently associated with clinical signs of disease; thus, the fish may be healthy carriers of the virus (Garseth, Fritsvold, Opheim, Skjerve, & Biering, 2013). The virus infects red blood cells (Finstad et al., 2014) and is distributed throughout the body's tissues via the circulation. The disease is characterized by necrosis and inflammation in the heart muscle and predominantly red skeletal muscle and has a high morbidity and up to 20% mortality (Kongtorp, Kjerstad, Taksdal, Guttvik, & Falk, 2004). Disease outbreaks are commonly observed after 5 to 7 months post-sea transfer (Kongtorop, Taksdal, & Lyngøy, 2004). Although the viral load increases during disease outbreaks, the virus can survive inside phagocytes and is therefore not eliminated, leaving the fish persistently infected (Malik et al., 2019). The inflammation of red skeletal muscle is characterized by necrosis of myocytes and infiltration of mononucleated cells. The white skeletal muscle may also be affected (Kongtorp et al., 2004a). In an experimental study on PRV-1 infection in rainbow trout, only mild pathological changes in the heart were detected, and persistent infection was not present (Purcell et al., 2020). In 2022, 147 outbreaks of HSMI in Atlantic salmon were reported (Olsen & Dahle, 2023). As the virus has not yet been cultivatable *in vitro* (Pham et al., 2020), attempts to develop efficient vaccines against infection have had moderate success, protecting against severe HSMI but not against PRV-1 infection (Haatveit et al., 2018; Wessel et al., 2018). Today, no vaccine against HSMI is utilized in Norwegian salmonid production.

Piscine orthoreovirus 3 (PRV-3)

PRV-1 infection can occur in rainbow trout, however, with only mild myocarditis in the heart observed (Purcell et al., 2020). In rainbow trout, HSMI is caused by another subtype of piscine orthoreovirus, PRV-3 (Olsen, Hjortaas, Tengs, Hellberg, & Johansen, 2015). This virus is closely related to PRV-1 with 89 % identity alignment (Dhamotharan et al., 2018) and can provide cross-protection against PRV-1 in Atlantic salmon (Malik et al., 2021c). This disease closely resembles HSMI in Atlantic salmon, yet there exist certain distinctions that set the two conditions apart. In rainbow trout, unlike in Atlantic salmon, HSMI is associated with anemia (Olsen et al., 2015). Additionally, the disease is usually observed in the freshwater stage of production, and the infection is more efficiently eliminated compared to PRV-1 in Atlantic salmon (Hauge et al., 2017; Vendramin et al., 2019). In Norway, PRV-3 was detected in ten localities in 2022. However, severe outbreaks of HSMI in rainbow trout have not been reported in the last decade (Olsen & Dahle, 2023).

Salmonid alphavirus (SAV)

Salmonid alphavirus is an enveloped single-stranded RNA virus belonging to the family *Togaviridae* (Nelson, McLoughlin, Rowley, Platten, & McCormick, 1995*)*. The subtypes SAV2 and SAV3 are the causative agents of pancreas disease (PD) in Atlantic salmon and rainbow trout (SAV2 and 3) and sleeping disease (SD) in rainbow trout (SAV2) (Hodneland, Bratland, Christie, Endresen, & Nylund, 2005; Nelson et al., 1995; Weston, Welsh, McLoughlin, & Todd, 1999). In Norway, there are currently two ongoing PD pandemics, geographically divided by the causative virus into areas north of Hustadvika, Møre and Romsdal (SAV2), and south of Stad, Vestland (SAV3) (Sindre, Patel, Olsen, & Løkslett, 2022). PD and SD are recognized by necrotic lesions and inflammation in the heart, red skeletal muscle, and the pancreas (Graham et al., 2007). Due to the characteristic pathological changes in the pancreas and the abnormal swimming behavior of the affected fish, likely caused by the compromised skeletal muscle, the disease has been named accordingly (McLoughlin & Graham, 2007). In Norway, the clinical signs occur in the seawater production phase in Atlantic salmon and rainbow trout, with a low to moderate mortality (Taksdal et al., 2007). An outbreak may last 3 to 4 months (Taksdal et al., 2007). However, the virus can be detected in recovered individuals (Andersen, Bratland, Hodneland, & Nylund, 2007). Although red skeletal muscle is more severely affected, with necrosis and inflammatory cell infiltration, white skeletal muscle can also show mild and severe degeneration (Taksdal et al., 2007). The virus can be detected in several tissues in infected fish (Andersen et al., 2007) and has been shown to target the satellite cells in the skeletal muscle of rainbow trout, likely explaining the degenerative properties in the tissue with a slow regeneration with macrophage infiltration and myofiber atrophy (Biacchesi et al., 2016; Heidari et al., 2015). Due to the lower prevalence of severe lesions in rainbow trout, Taksdal et al. 2007 discussed whether rainbow trout may be less susceptible to SAV infection than Atlantic salmon or if the regenerative process is more successful. In 2022, 90 outbreaks of PD were reported in Norway (Sindre et al., 2023). Several vaccines are available and utilized in Norwegian salmonid production, however with some variations in protective properties (Jensen, Kristoffersen, Myr, & Brun, 2012; Karlsen et al., 2012; Veenstra et al., 2020).

5.4 Red and melanized focal changes in farmed Atlantic salmon and rainbow trout

5.4.1 Prevalence and concerns in the industry

In farmed Atlantic salmon in Norway, the findings of the fillet's red and melanized focal changes (RFC and MFC), commonly known as red and dark spots, have increased during the last three decades (Mørkøre et al., 2015). Today, the prevalence of MFC in slaughter-sized fish is, on average, 20-30 % (Bjørgen et al., 2019; Mørkøre et al., 2015), and the discoloration is detected at slaughter. MFC have not been observed in the freshwater phase of production but show a steady increase in prevalence after transfer to seawater (Bjørgen et al., 2019). RFC, on the other hand, has been reported in freshwater smolts (Jiménez-Guerrero et al., 2022) and has a steady prevalence of 1-5 % with no increase during the seawater phase (Bjørgen et al., 2019). RFC and MFC have not been reported in wild Atlantic salmon. As for farmed rainbow trout, the prevalence of MFC is reported to be much lower in slaughter-ready fish, with an average prevalence of 1-2 % (Mørkøre et al., 2015). The changes predominantly occur in the cranio-ventral and cranio-dorsal part of the abdominal wall, primarily seen on the peritoneal side of the fillet (Fig. 7), but with the potential to reach through the skeletal muscle to the skin. The changes result in the cassation of the affected area. As a result, RFC and MFC cause great economic losses for the Atlantic salmon industry.

5.4.2 Characterization of changes

To utilize a suitable surveillance system regarding the prevalence of the changes, several classification systems have been developed for use during visual evaluation of the fillet at slaughter. The changes vary in size and color intensity; thus, the macroscopic evaluation is based on their localization and appearance. One of Norway's largest seafood companies, Mowi, has developed a classification system grading the RFC and MFC with increasing severity, from grade 1 (small focal change and light discoloration) to grade 3 (large change with intense discoloration) (Bjørgen et al., 2019). This grading system is widely used in the industry and during research investigations on the changes. In a report from The Norwegian Institute of Food, Fisheries and Aquaculture Research (Nofima), another system was developed to evaluate the changes, including the localization (anterior abdominal wall, posterior abdominal wall, and dorsal musculature) and severity (grade 1, 2, 4 and 8) (Mørkøre, 2012). Grade 1 includes changes with only weak discoloration, grade 2 represents focal changes smaller than 3 cm, grade 4 includes changes within 3-6 cm sizes, and grade 8 is given to melanized areas larger than 8 cm. Recently, an additional method has been developed to evaluate melanized changes in the fillet while adjusting for fish size (Jiménez-Guerrero et al., 2022).

Descriptive studies on RFC and MFC histological properties in Atlantic salmon have also been performed. RFC presents characteristic pathological changes in line with acute inflammation, including hemorrhage and necrotic myocytes (Fig. 7) (Bjørgen et al., 2019; Bjørgen et al., 2020). MFC, on the other hand, represents a chronic, granulomatous inflammation in the skeletal muscle (Fig. 7), with organized granulomas being a frequent finding (Koppang et al., 2005; Larsen et al., 2012). Categorization of the histological findings in MFC in Atlantic salmon has been developed, grading the microscopic changes in the skeletal muscle from 1 to 9 (Bjørgen et al., 2019). Category 1 includes samples with no pathological observations; category 2 represents intact myocytes with infiltration of endomysial melano-macrophages; category 3 represents intact myocytes with surrounding fibrosis,

and no melano-macrophages, category 4 includes the changes of category 3 with the presence of melano-macrophages, category 5 represents samples with more abundant inflammation and fibrosis in the tissue, while category 6 and 7 includes samples with larger areas of fibrosis and inflammatory cells, with and without melano-macrophages, respectively. Category 8 represents inflammation with organized granulomas, whereas category 9 shows diffuse granulomatous inflammation in larger areas of the skeletal muscle. It is shown that the histological category of the MFC corresponds to the macroscopic grade of the changes using the Mowi classification system; increasing macroscopic grades have a higher histological category, however with a significant variation of histological categories found in grade 1 MFC (Bjørgen et al., 2019). No protocol on the development from RFC to MFC is established, however, given their typical localization, the histological findings on acute inflammation in RFC and chronic inflammation in MFC, the finding of transition forms between RFC and MFC (Bjørgen et al. 2019), in addition to the steady prevalence of RFC as opposed to the increasing, accumulative prevalence of MFC, the assumption that RFC develops into MFC is valid.

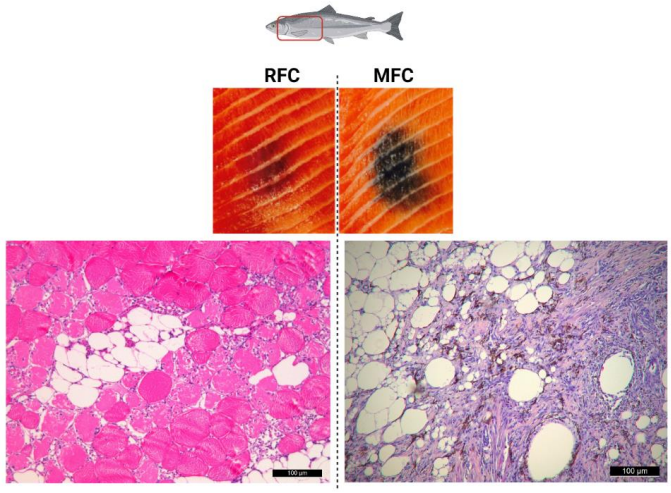

Figure 7. RFC and MFC in Atlantic salmon. The changes are predominantly observed in the cranioventral region of the abdominal wall. The macroscopic focal red change is characterized histologically by hemorrhage and necrotic myocytes (Scale bar = 100 µm). In contrast, the melanized focal changes in severe cases represent a chronic granulomatous inflammation with vacuoles and an abundance of infiltrated melano-macrophages (Scale bar = 100 µm). Created with BioRender.com.

5.4.3 Aetiology: Current knowledge

Being the reason for significant economic losses in the industry, the cause of RFC and MFC have been given increasing interest during the last decades. Many theories have been launched regarding the initiating factor (Fig. 8). One of the first hypotheses regarding the cause and development of MFC in farmed Atlantic salmon was the role of intraperitoneally injected adjuvanted vaccines (Koppang et al., 2005). Rightfully, due to intraperitoneal injections, local inflammation is initiated, affecting the abdominal organs and the soft tissue in the abdominal cavity (Mutoloki et al., 2006; Roberts, MacQueen, Shearer, & Young, 1973a, 1973b). Accordingly, development of granulomas can occur in many fish species as a result of injected foreign materials (Balouet & Laurencin, 1986), including the presence of melanin-containing cells (Roberts et al., 1973b). The findings of large, lipid-

containing vacuoles in MFC were suspected to result from the oil adjuvants utilized in the vaccines (Koppang et al., 2005; Larsen et al., 2012). The correlation between vaccine injections and the development of MFC is thus reasonable. However, MFC has also been reported in unvaccinated fish (Larsen et al., 2014; Berg et al., 2007), indicating additional factors to influence the observed changes. The increasing prevalence of MFC seen during the last three decades correlates with important changes in commercial fish feed, from fish oil to vegetable lipid sources. As mentioned earlier, the nutritional impact on the immune system is evident, as anti-inflammatory oils are exchanged for proinflammatory substances (Montero et al., 2010). A low level of EPA and DHA in fish feeds has been reported to increase the prevalence of MFC (Sissener et al., 2016). Additionally, the use of antioxidants, and to some degree zinc, has proven to reduce the number of MFC compared to control feeds (Mørkøre et al., 2015).

Another possible cause of acute inflammation in the skeletal muscle is external trauma, causing local damage in the tissue. During salmonid production, the fish are handled during transportation, vaccination, and medical and mechanical treatment of infection and disease, typically using pumping devices. An increase in MFC has been observed during external trauma (Mørkøre et al., 2022); however, the consequences of traumatic incidents in salmonid farming and possible harmful effect on both the axial skeleton and the skeletal muscle is not well studied. The handling of the fish is also associated with low oxygen levels in the water due to stressful impacts on the fish. Low oxygen levels have been shown to affect the prevalence of MFC negatively (Mørkøre et al., 2015). Hypoxia in the local tissue due to vascular pathologies such as thrombosis has also been speculated to cause necrosis and hemorrhage. Additionally, as an acute inflammatory reaction is commonly solved efficiently by the immune system under normal physiological and metabolic conditions, the suggestion that the acute inflammation (RFC) in the skeletal muscle is developing into a chronic granulomatous inflammation (MFC) has shed light on both the rate and mechanism behind the regenerative process and also the possible presence of a persistent agent in the tissue.

First, the intriguing fact that RFC and MFC have not been observed in wild salmon raises the question of whether the chronic changes result from genetic divergencies in immune responses between the farmed salmon and its wild, non-bred counterpart. The same question is valid, looking at the different prevalence of the changes in farmed Atlantic salmon versus farmed rainbow trout, despite similar production procedures in both species. Detailed analysis of these questions has not yet been performed. As for the presence of persistent agents, several pathogens have been identified and suggested to interfere with the development of MFC, particularly the viruses affecting the skeletal muscle in salmonids such as PRV-1 and SAV. However, only the presence of PRV-1 in severe focal changes has been shown to have a plausible effect on the ongoing chronic inflammation (Bjørgen et al., 2015; Malik et al., 2021a). As RFC and some MFC can be found without PRV-1, the initial cause of the changes must lie elsewhere.

Altogether, the causative factors of RFC and MFC can be numerous. By studying the independent factors that might contribute to the development of the changes, one may reveal the significance of each of the separate factors. In addition, by describing other types of muscle melanization and even melanization in different species, one may acquire important knowledge on the initial cause and the development of the condition.

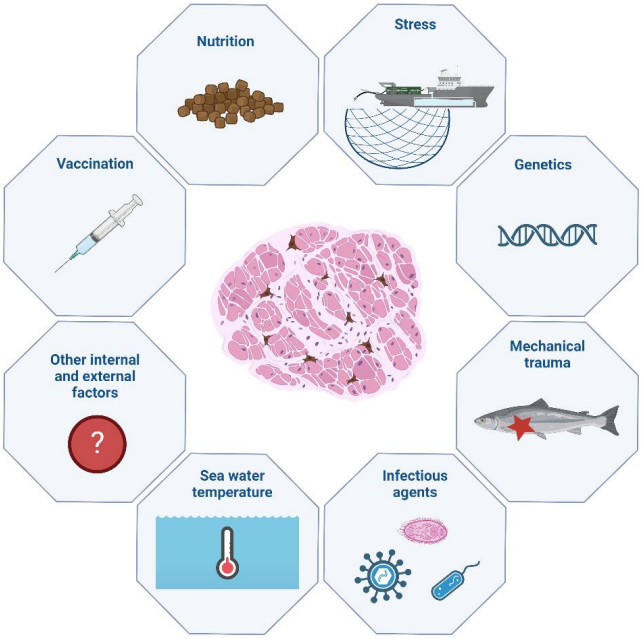

Figure 8. Possible causative factors influencing the development of RFC and MFC. Vaccine side-effects, nutritional properties, stress-related situations with low oxygen levels, genetic divergencies, mechanical trauma, infectious agents, sea water temperature effect on regeneration, and other possible external factors have all been suggested, but no single cause has been detected. Created with BioRender.com.

5.5 Aims and objectives

The ultimate research goal of this thesis was to investigate and describe potential causative factors regarding the melanization of the skeletal muscle in farmed Atlantic salmon and rainbow trout. This was done by studying independent factors that might contribute to the development of red and melanized changes, and thus reveal the significance of each of the separate factors, and by describing and comparing other types of muscle melanization and melanization in different origin groups and species.

To approach the research goal, several hypotheses were postulated to provide further knowledge on the matter:

Hypothesis 1: Red and melanized focal changes are associated with pathological conditions in the ribs

Hypothesis 2: Melanized changes in skeletal muscle are similar in both red and white skeletal muscle

Hypothesis 3: Prevalence and development of melanized changes are influenced by the genetic differences between farmed and wild Atlantic salmon

Hypothesis 4: Prevalence and morphological characteristics of melanized focal changes in farmed rainbow trout are similar to that in Atlantic salmon.

As ribs are closely related to the skeletal muscle. i.e., the fillet, it was of interest to reveal if pathological conditions in the ribs could be associated with melanized focal changes in the white skeletal muscle. The aim of paper I was to investigate this hypothesis and thus provide new knowledge on causative factors for the development of the condition in relation to pathological changes in the ribs. Paper II aimed to describe histological properties and infectious agents present in diffusely melanized red skeletal muscle, an atypical melanized change in Atlantic salmon. Uncovering possible differences could contribute to additional knowledge on the development of the changes. Also, as genetic differences are known to affect both physiological and immunological responses, and as RFC and MFC have not been observed in wild Atlantic salmon and with a lower prevalence in farmed rainbow trout, paper III and IV aimed to test the related hypothesis, investigating the prevalence and regeneration of RFC and MFC in both Atlantic salmon with different origin (farmed, hybrid, and wild), and in different salmonid species (Atlantic salmon and rainbow trout).

6 MATERIALS AND METHODS

6.1 Materials

For this thesis, farmed, wild, and hybrid Atlantic salmon and farmed rainbow trout were sampled based on the presence of RFC and MFC. The samples were retrieved from commercial Norwegian producers, research facilities, and rivers in Norway (Fig. 9). All handling of the fish, including anesthesia and euthanasia, was conducted according to EU and Norwegian legislation. Legislation references are summarized in figure 9.

In paper I, fillets from farmed Atlantic salmon were evaluated at slaughter at the facilities of Bremnes Seashore AS, a commercial aquacultural producer in Bremnes, Norway. Wild-caught spawning Atlantic salmon was sampled from the river Drammenselven in Viken, Norway. Samples were also retrieved from Matre Research Station in Matre, Norway, representing wild, hybrid, and farmed Atlantic salmon. Material from the latter was also used in paper III. The samples for paper I included white skeletal muscle, ribs, and peritoneum. Selection criteria were the presence of red and melanized focal changes and no discoloration (controls) found in areas adjacent to the ribs in the abdominal cavity. In paper II, whole-side fillets from farmed Atlantic salmon containing red and white skeletal muscle were sampled at slaughter. The samples were retrieved from a commercial producer, Lerøy Seafood Group ASA location at Kjørsvikgrunn in northwestern Norway. The skin was removed at slaughter, revealing the discoloration of the red skeletal muscle. For this sampling, the selection criteria were the presence of diffuse melanization of red skeletal muscle. Samples with no discoloration from the same fish population were used as controls.

The material used in paper III was also partly used in paper I. Atlantic salmon from farmed (bred), hybrid, and wild origin were kept in identical rearing conditions from hatching to slaughter at the facilities of Matre Research Station. As this material was part of an animal study, the research was reviewed and approved by the Animal Care and Use Committee/IACUC and NARA (permit number 15780) according to the European Union Directive2010/63/EU and Norwegian regulation FOR-2015-06-18-761. The fish were macroscopically analyzed for red and melanized focal changes from the late freshwater stage and throughout the seawater stage. Random selection for measuring production parameters (growth, length, etc) was also performed throughout the trial. RFC and MFC in the abdominal wall were the selection criteria for further histological investigations.

Paper IV included fillets from farmed rainbow trout from three farms in Western Norway. The selection criteria were the presence of red or melanized changes in the white skeletal muscle of the fillet at slaughter.

Paper	Species	Producer	Legislation for handling
Paper I	 Atlantic salmon	Bremnes Seashore AS Matre Research Station* Wild caught salmon	Akvakulturdriftsforskriften §34 Avliving av fisk (Lovdata, 2018)
Paper II	 Atlantic salmon	Lerøy Midt AS (Kjørvikgrunn)	Akvakulturdriftsforskriften §34 Avliving av fisk (Lovdata, 2018)
Paper III	 Atlantic salmon	Matre Research Station*	Approved by the Animal Care and Use Committee/IACUC and NARA (permit number 15780) according to the European Union Directive2010/63/EU and Norwegian regulation FOR-2015-06-18-761
Paper IV	 Rainbow trout	Lerøy Vest AS/Sjøtroll Havbruk AS (Tepstad) Lerøy Vest AS/Sjøtroll Havbruk AS (Djupestallen) Blom Fiskeoppdrett (Laksevika)	Akvakulturdriftsforskriften §34 Avliving av fisk (Lovdata, 2018)

*Figure 9. Material used in the thesis. For paper I-III, Atlantic salmon was used. In paper I, fish were obtained from a commercial producer, research facilities, and a river with spawning Atlantic salmon. For paper IV, rainbow trout from three different locations were investigated. Legislation references for handling of the fish are included.*Fish from Matre research station were used in papers I and III. Created with BioRender.com.*

6.2 Methodology

Several methodological techniques were utilized to approach the hypotheses postulated in this thesis. Radiographic, histological, and genetic analyses were performed.

6.2.1 Radiography

Radiographs of selected fillets with a focus on the ribs to investigate skeletal abnormalities were performed in paper I. Specific settings were applied according to tissue size and to highlight bone (high milliampere/second (mAs), low kilo voltage (kV) technique) using a direct digital system (SoundEklineSeries DR). The size of the samples ranged from 5 x 6 cm to 10 x 15 cm. For the small samples, 9 mAs and 40 kV were used; for the larger samples, 16 mAs and 40 kV were used.

6.2.2 Histology

The use of histological examinations was vital for further describing the findings of the radiographic experiments, but also to accompany current knowledge on microscopic details regarding RFC and MFC and provide further information on the matter. Samples were embedded in paraffin for all histological procedures according to standard protocols. In paper I, the samples included bone tissue. Thus, decalcification was necessary, as this process enables optimal sectioning of bone tissue. After radiographic examinations, the samples were decalcified in a 0.5 M pH 8 EDTA solution (disodium ethylenediaminetetraacetate, 2H2O) before further dissection for block preparation. During this process, which occurs after fixation, minerals are removed from the bone. This can be done using strong mineral acids, weaker organic acids, or chelating components (Rolls, 2023). Chelating agents, such as EDTA, are the gentlest approach and most suitable for detailed examination and advanced histological techniques such as *in situ* hybridization. Here, the hydroxyapatite crystals in the bone are reduced in size by removing surface calcium.

In this thesis, several stains were used to detect specific properties in the tissue, including hematoxylin and eosin stain (HE) for nuclear and cellular components, Lendrums Maritus Scarlet Blue stain (MSB) for fibrin, Giemsa stain for nuclear and cytoplasmic morphology and parasite deoxyribonucleic acid (DNA) and Gram stain for bacterial detection. Van Gieson stain (VG) for collagen fibers and connective tissue and Masson Trichrome stain (MT) for distinguishing collagen and connecting tissue from adjacent cells (Padmapriya & Lakshmi, 2021), Fontana Masson stain (FM) for melanin was also conducted (Churukian, 2008).

6.2.3 Reverse transcription quantitative polymerase chain reaction (Rt-qPCR)

Reverse transcription quantitative polymerase chain reaction (Rt-qPCR) is used for both qualitative and quantitative analyses of detecting specific nucleic acid in tissues. qPCR is the preferred method for detecting the presence and amount of virus in specific tissues. During the detection of RNA viruses, the ribonucleic acid (RNA) is transcribed into DNA through reversed transcription, which is further analyzed through light cycle procedures. The DNA sequence is amplified, and by using sequence-specific primers, adding fluorescence dye (the SYBR Green method) (Fig. 10), or fluorescent probes (the TaqMan method), the increased fluorescence signal corresponds with an increasing amount of viral-RNA present at each cycle. The cycle threshold (Ct) value represents the number of cycles necessary for detecting the fluorescent signal over background levels. Thus, the lower the Ct value, the more abundant viral nucleic acid present. The test is regarded as negative above a specific number of cycles, i.e., a particular Ct value.

A RT-qPCR method conducted by PatoGen was utilized in all four papers to detect the presence of viral genes in the tissue. PatoGen meets the requirements of Norwegian Standard NS-EN ISO/IEC 17025, 'General requirements for the competence of testing and calibration laboratories'. In papers I and III, the spleen was used to detect virus in fish from Matre Research Station and the wild-caught fish, while in paper II and IV, the skeletal muscle was used. In paper IV, the heart was also utilized for RT-qPCR analyses. Samples selected for RT-qPCR analysis were transferred to RNAlater after dissection. The samples were sent to PatoGen AS, Ålesund, Norway, for the Rt-qPCR analysis. The analyses are accredited and validated to ISO7025 standards.

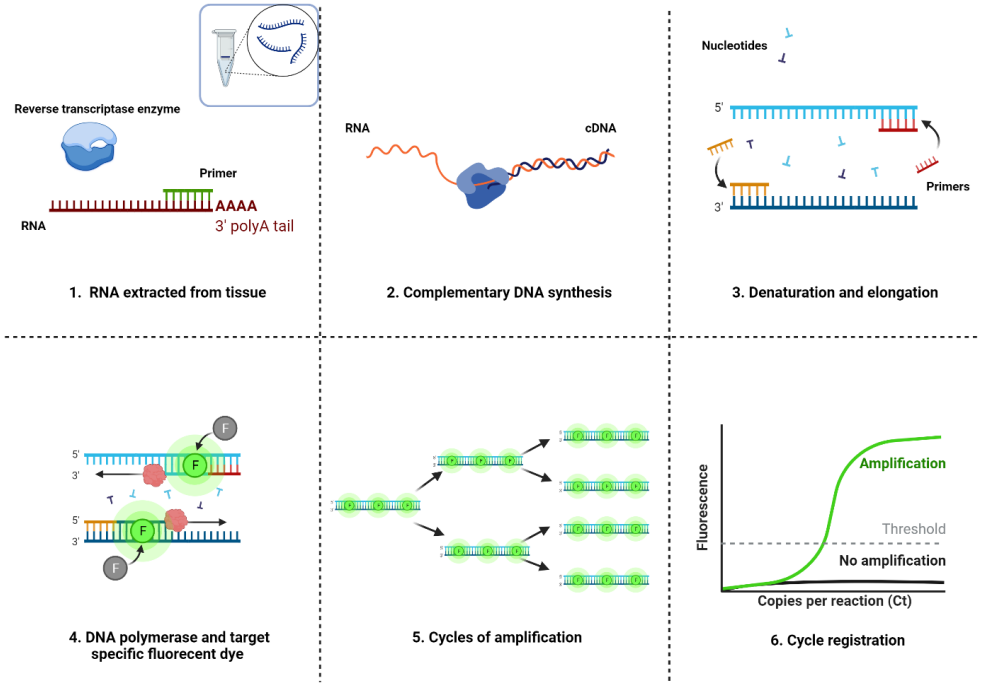

Figure. 10. Reverse transcriptase qPCR illustrated using the SYBR Green method. RNA extracted from the tissue is synthesized to complementary DNA(cDNA) by reverse transcriptase enzyme. Following denaturation of cDNA, primers attach, and through DNA polymerase, nucleotides and target-specific fluorescent dye creates double-stranded DNA molecules. The fluorescent signal is detected through amplification cycles, resulting in a Ct value representing the number of cycles acquired for detecting the signal. Created with BioRender.com.

6.2.4 *In situ* hybridization (ISH)

Using the *in situ* hybridization method, as the name implies, one can detect the presence and localization of specific DNA or RNA sequences in the tissue. To detect the messenger RNA (mRNA), a complementary probe linked to a fluorescent molecule or a chromogen enzyme creates a signal that is detectible in a microscope (Fig.11). In this thesis, a specific method of in situ hybridization known as RNAscope was utilized. Here, specific RNAprobes are designed to detect target RNA in the tissue. An initial fixation and permeabilization procedure makes the tissue available for the probes. The standardized probe design includes up to 20 highly specific Z-probes. Although up to 20 probes can be present, only three bindings are acquired for signal visualization. Additionally, only one RNA transcript in the tissue can create detectable signals, making this method highly specific and sensitive. Each Z-probe consists of three elements: the bottom part is complementary to the target RNA, the top part represents the binding site for a preamplifier, and a mid-part binds the bottom and top part together. To successfully create a binding site for the preamplifier, two Z-probes must hybridize to the target sequence in tandem. After the preamplifier attachment, multiple amplifiers will hybridize to the preamplifier, followed by the adhesion of fluorescent molecules or chromogen enzymes. The labeled RNA can be visualized using a standard light microscope (with the chromogen), making it possible to visualize both the target RNA's presence and localization.

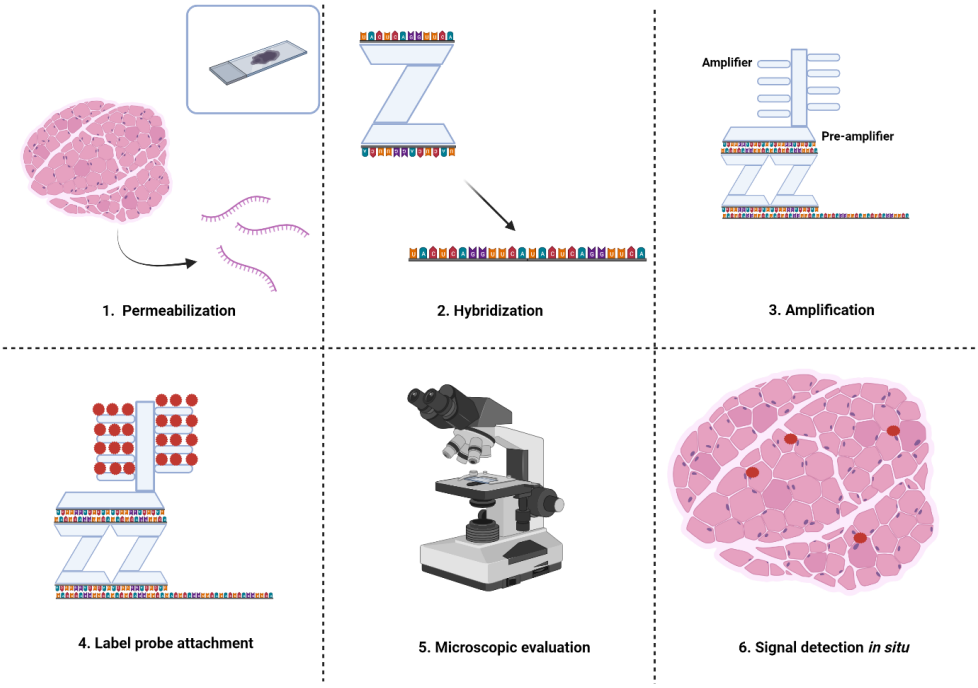

Figure 11. In situ hybridization. After permeabilization of the tissue, a complementary Z-probe attaches to a specific sequence in the RNA molecule. When two Z-probes hybridize to the target sequence, the preamplifier attach, followed by the attachment of amplifiers. The adhesion of chromogen enzymes to the amplifiers visualizes the localization of the RNA sequence in the tissue with a light microscope. Created with BioRender.com.

7 SUMMARY OF SEPARATE PAPERS

Paper I:

The specific localization of RFC and MFC in the cranio-ventral and cranio-dorsal part of the abdominal wall in 90 % of the fish indicates a site-specific causative factor for the changes. In this anatomical area, the ribs are located beneath the peritoneum. Thus, pathological conditions in the ribs, specifically trauma and fractures, are valid factors to explore concerning RFC and MFC. In paper, I, 129 samples were selected based on the presence of RFC and MFC at the time of slaughter, including equivalent areas of the fish selected for controls. To investigate if the adjacent ribs were involved in the change, both ribs and peritoneum were retained in the samples. Through radiographic and histological investigations, variations within costal morphology and vascularization in Atlantic salmon were detected and described, providing further knowledge on anatomical properties and, importantly, giving more solid information on putative non-pathological morphology when examining ribs in relation to adjacent soft tissue inflammation. Costal abnormalities detected in radiographic investigations, such as mild rib axis deviation, ribs with wave-shaped distal ends, and ribs with a radiolucent medulla, showed no statistical association with macroscopic discoloration or histological changes in the skeletal muscle; thus, these costal variations were suspected to be non-pathological. The results revealed a statistically significant association between costal fractures and RFC and MFC. A statistically significant association was also found between MFC and callus formation in the ribs. These results, combined with the vascularization of the ribs, implies that a costal fracture in Atlantic salmon may cause a local hemorrhage and acute inflammation in the adjacent soft tissue (seen as RFC). The acute inflammation is followed by reparation and later bone and soft tissue regeneration, resulting in callus formation, as seen in mammals. Thus, the association between callus formation and the more progressed changes (MFC), but not RFC, is valid. However, RFC and MFC were also seen without any sign of costal interference, implying that other factors may be involved in the development of these focal changes. In conclusion, costal fractures in farmed Atlantic salmon are likely to evoke an acute reaction in the neighboring soft tissue and can be resolved in a manner similar to fracture healing in mammals. The development of MFC is suggested to result from a persistent factor driving chronic inflammation, as previously suggested (Bjørgen et al., 2019; Malik et al., 2021a), possibly also impairing bone tissue repair. Conversely, chronic inflammation in the soft tissue caused by alternative factors may affect the adjacent bone (Epsley et al., 2021; Kawao & Kaji, 2015), resulting in poor bone health and, consequently, fractures.

Paper II (Short communication):

As previously described, the majority of RFC and MFC in farmed Atlantic salmon and rainbow trout are localized in the abdominal wall towards the peritoneal side of the truncus. However, melanization has been observed in other areas as focal, distinct discolorations and as more diffuse discoloration in the dorsal skeletal muscle (Mørkøre et al., 2015). Other localizations, and even more so, different tissue distributions (focal or diffuse), point to potentially different causative factors. Paper II focused on the morphological and histological properties of diffuse melanization in red skeletal muscle in farmed Atlantic salmon to investigate if non-typical melanization differs from MFC. Six whole-fish fillets with red skeletal muscle melanization were examined, and six fillets without discoloration from the same population were used as controls. The samples were obtained from Lerøy Seafood Group ASA, a commercial producer in Norway. Both the localization and distribution of

melanin differed from the common MFC. The affected fish contained dark, melanized red skeletal muscle involving the entire red muscle mass. There was a clear demarcation zone between the affected red skeletal muscle and the unaffected white skeletal muscle, seen both macroscopically and microscopically. Histological investigations, including special stains, revealed diffuse inflammation with degenerative and necrotic muscle fibers interspersed between large amounts of fibrotic tissue and, importantly, an abundance of melano-macrophages. Some muscle regeneration was also observed. Both Giemsa and Gram staining were negative. Altogether, the histological findings were similar to that commonly seen in severe MFC in white skeletal muscle, however, organized granulomas were not observed. This may be due to a limited number of samples, a dissimilar inflammatory reaction in red versus white skeletal muscle due to differences in metabolic and vascular characteristics, or a different initial and/or persistent causative factor in diffuse red muscle melanization compared to white muscle MFC. Both PRV-1 and SAV2 may induce red skeletal muscle inflammation, however, SAV2 was only detected in three of the six affected fish. The presence of PRV-1 was statistically higher in the affected samples compared to the controls, although the virus was detected in 10 of 12 samples by RT-qPCR. Taken together, the diffuse melanization and the presence of PRV-1 suggest a potential association between PRV-1 and the onset of this condition. This seems especially plausible considering the common occurrence of red skeletal muscle affection seen in cases of HSMI caused by PRV-1.

Paper III:

Although RFC and MFC are prevalent in the production of Atlantic salmon, such changes have not been observed in wild Atlantic salmon. This could result from genetic differences in wild versus farmed and bred salmon or may be linked to environmental factors associated with production practices. In paper III, 1854, Atlantic salmon of farmed, hybrid, and wild origin were reared under identical conditions from hatching to slaughter. The presence and prevalence of RFC and MFC were investigated at three time points: Once prior to sea transfer and at two time points after sea transfer. No macroscopic discoloration was detected at the freshwater stage; however, both RFC and MFC occurred after sea transfer. RFC showed a steady prevalence throughout the trial (1-5 %), whereas MFC showed an increase from sea transfer to slaughter (20-30 % at slaughter), both coinciding with findings in earlier reports on the prevalence of RFC and MFC in farmed Atlantic salmon (Bjørgen et al., 2019). The presence of PRV-1 was detected in the spleen in 98% of the samples selected for RT-qPCR during the seawater phase. There were no statistical differences between the origin groups regarding the presence of RFC and MFC. These results implied that the genetic factor, i.e., differences in origin, is not a causative or developmental factor for the occurrence of RFC and MFC in Atlantic salmon. Histological examinations also showed similar inflammatory reactions in the skeletal muscle and adipose tissue in fish from all origin groups. This suggests that external factors are likely the primary contributors to these observations. In addition to these results, histological investigations, including the Fontana Masson stain for melanin, revealed the presence of melano-macrophages in the adipose tissue in freshwater smolts prior to sea transfer. These findings, including the abundance of melano-macrophages in the adipose tissue in several samples from the seawater phase, suggests a link between steatitis and the development of MFC.

Paper IV:

Having ruled out genetic divergences as a potential causative factor for RFC and MFC in Atlantic salmon in Paper III, it became crucial to investigate whether the same held true for rainbow trout.

Although the observed prevalence of RFC and MFC in farmed rainbow trout is lower, there are no histological descriptions of the changes in farmed rainbow trout. In paper IV, samples from a total of 1293 fish were retrieved from three different commercial farms in Norway. The presence and severity of RFC and MFC were registered at slaughter, and samples for histological examinations and RT-qPCR were selected based on the presence of the changes. The prevalence of MFC varied from 1,46% in farm 1, 6,47% in farm 2, and 5,76% in farm 3. RFC was found in only one fish in farm 1. The changes were primarily localized in the cranioventral region of the abdominal wall and typically of low severity. Histological examinations revealed some common characteristics with MFC in Atlantic salmon. However, notable differences were observed, such as a relatively low prevalence of melano-macrophages and organized granulomas. RT-qPCR revealed the presence of both PRV-1 and SAV in skeletal muscle and PRV-3 in the heart; however, *in situ* hybridization (ISH) showed no relation to the lesioned areas in the tissue. In light of the macroscopic localization of RFC and MFC, their lower prevalence compared to Atlantic salmon, and the histological distinctions from Atlantic salmon MFC, our findings suggest that the underlying causes of RFC and MFC in rainbow trout are similar to those in Atlantic salmon. However, the inflammatory response and regenerative capacity appear to differ, possibly owing to distinct reactions to viral infections.

8 DISCUSSION

8.1 Material and methodological considerations

8.1.1 Macroscopic grading systems, radiography, and histology

The selection of materials for this thesis aimed to provide a comprehensive overview of RFC and MFC in the commercial production of Atlantic Salmon and rainbow trout in Norway. This was done by retrieving samples from different production sites and conducting the sampling during standard slaughter procedures, except for the fish in paper III. This diverse sampling approach enabled us to capture a broad representation of changes, ensuring that our findings could be generalized across different production locations, thus minimizing potential biases related to locality differences. A drawback of collecting samples from different locations is the variability in the assessment of the macroscopic discoloration in the fillet, which can be influenced by different personnel conducting the evaluations. In paper I-III, macroscopic evaluation was performed by the local staff at the abattoirs using the grading system developed by Mowi, grading RFC and MFC from 1 to 3. Although this is a common system used by the industry, detailed registrations of the changes are left out. A specific change may have varying appearances as a result of vaccine side effects/erroneous injection, trauma, or other causes. However, distinguishing and standardizing these changes can be challenging using a three-grade system based on color intensity and size. The grading system developed by NOFIMA utilized in paper IV includes four grades, thus more detailed but still challenging to use when attempting to differentiate between possible causative factors. Importantly, the low-severity grades (Mowi grade 1: Small focal change and light discoloration, and Nofima grade 1: Weak discoloration, and grade 2: Focal changes smaller than 3 cm) might be challenging to detect for untrained observers or during fast-paced slaughter procedures. Specifically, RFC grade 1 in the skeletal muscle can be challenging to distinguish from a small hemorrhage in the peritoneum without the use of more detailed examination or even histological examinations. Consequently, the grading system may not consistently yield standardized assessments, especially when applied by different handlers and personnel. Importantly, using different grading systems makes it challenging to compare results when looking at the presence and variations in severity of the grades in this thesis. Therefore, despite the existence of several grading systems, the industry would benefit from using a single, standard grading system to provide a more accurate assessment of the prevalence of RFC and MFC.

In paper I, RFC and MFC were evaluated combined with radiographic examinations aiming to reveal abnormal ribs in the area of interest. This approach provided valuable insight into both the potential involvement of the ribs in the observed macroscopic changes and the prevalence of costal abnormalities within these samples. Radiographic investigations on fish ribs have been successful in earlier reports, revealing bone fractures and callus formations (Fjelldal et al., 2018). Spatial resolution of radiographs is higher than computed tomography (CT), but the latter with the advantage of providing further information on the 3D structures of the examined tissue. However, such methods would be both more expensive and time-consuming. Importantly, histological examinations accompanying the radiograph findings proved very important during the work on paper I and should not be replaced by advanced imaging modalities. This was illustrated by the finding of fractured ribs on radiographs, which, upon histological analysis, revealed no signs of soft tissue inflammation adjacent to the fractures. This indicates that the fracture likely occurred

postmortem, a possibility that was not unlikely in our study due to the handling of the fillets during separation from the vertebral spine. In a statistical analysis of the prevalence of costal fractures, these postmortem fractures could potentially contribute to erroneous results if not revealed by histology. This should be considered in future radiographic investigations on costal fractures in fish. Moreover, in paper I, RFC and MFC could be detected in relation to costal abnormalities other than fractures by radiographic imaging; however, histological investigations revealed costal morphology with putatively normal variations devoid of adjacent soft tissue inflammation, suggesting that the RFC/MFC was not related to the specific costal abnormality in question. All in all, without histological investigations complemented by a thorough understanding of the normal variations in costal morphology, the prevalence of costal fractures in live fish and the correlation between RFC/MFC and costal abnormalities can be misinterpreted.

When detecting the presence of melanin in melano-macrophages, the Fontana Masson stain was utilized in paper III and IV. Other pigments, such as lipofuscin and hemosiderin, are also known to be present in these cells. In HE stained sections, melanin and lipofuscin are particularly difficult to distinguish, as they both hold brown/black color. Specific special stains are developed for lipofuscin and hemosiderin, such as Sudan Black stain and Perls' Prussion blue stain, respectively. During the work of paper III, sections were stained for lipofuscin in addition to melanin using Sudan Black, however the stain gave little additional information as the color of the pigment remained the same in both stains, making it challenging to distinguish melanin from lipofuscin. However, one cannot rule out the presence of lipofuscin based only on positive staining for melanin. Other staining protocols could be added to differentiate melanin and lipofuscin, such as the Nile Blue method (Lillie, 1956). However, due to the positive staining for melanin, the use of further stains for lipofuscin proved unnecessary for the purpose of this thesis.

In paper II and IV, special staining for the detection of collagen fibers were used. In paper II, the VG stain showed abundant collagen deposition, indicating fibrosis in the tissue. In paper IV, both VG and MT stains were utilized to detect possible differences in results between the two staining procedures. Both stains are widely used for the detection of collagen fibers; however, utilizing three colors, MT stain can differentiate important histopathological structures and is thus a common stain during tissue analyses in disease and, importantly, in myopathological conditions (Dubuisson et al., 2022; Kalpajyoti, Girish, Sanjay, Alshame, & Dinesh, 2018). Both stains have similar limitations when it comes to the identification of thin collagen fibers. However, the revealed collagen in paper II and IV indicated that the amount of collagen in these samples exceeded the minimal detection level. Additionally, the level and organization of collagen fibers in paper IV was similar in both stains, indicating that the use of one of the stains is sufficient when detecting connective tissue during scar formation and fibrosis in the skeletal muscle of salmonids.

8.1.2 RT-qPCR and ISH

The use of RT-qPCR in our research enabled the determination of the presence of specific viral RNA in our material. Given previous reports linking severe MFC to the PRV-1 virus, identifying this virus in our material could add additional weight to these results. Other viral infections could also contribute to the discussion regarding possible co-infections and their implications. In paper I, RT-qPCR was only conducted on spleen from the wild-caught fish and selected fish from Matre Research station. The rest of the material in paper I was diagnosed based on clinical manifestations of HSMI during production. Thus, the presence of PRV-1 in the skeletal muscle was not investigated by RT-qPCR in

paper I and III; however, the focus in papers I and III was primarily to investigate correlations between costal abnormalities and RFC/MFC and genetic differences affecting their morphology and prevalence, respectively. Additionally, RT-qPCR does not detect the specific location of the viral RNA within the tissue. Thus, a positive result for PRV-1 in skeletal muscle in paper I and III would not have yielded sufficient knowledge about the virus's contribution to RFC/MFC, as PRV-1 infects red blood cells and can be present in organs and tissues throughout the body. This is also true for using RT-qPCR in skeletal muscle in paper II. In contrast, ISH would be the most suitable method to detect and localize viral nucleic acids in the tissue. In paper I, attempts were made to conduct ISH on the samples. This proved unsuccessful, likely due to the length of the fixation time of the samples. For ISH by RNAscope, fixation should be restricted to 72h, and in our samples, longer fixation was necessary due to large sample sizes. The samples were also decalcified in 0.5 M pH 8 EDTA solution for a minimum of five days; however, as EDTA is a slow but gentle method of decalcification, the duration of this process is less likely to affect the RNA quality in the specimens. Both formalin fixation, within the appropriate duration, and EDTA decalcification have been shown to preserve sufficient amounts of RNA in the tissue to successfully conduct ISH, both in paraffin-embedded samples (Walsh, Freemont, & Hoyland, 1993) and resin-embedded blocks (Huysseune, Soenens, & Witten, 2021).

An interesting discussion regarding the virus localization in the material in paper I is the possible presence of PRV-1 in the osteoclasts. It has been demonstrated that PRV-1 persistently infects macrophages (Malik et al., 2019; Malik et al., 2021a). In mammals, the hematopoietic-originating osteoclasts share the same origin as macrophages (Bar-Shavit, 2007; Jacome-Galarza et al., 2019) and possess the ability to serve as APCs with expression of MHC class I and II (Li et al., 2010). Thus, persistently PRV-1 infected osteoclasts in salmonids may express M1-polarization and therefore show increased resorption activity, as seen in other bone-related viral diseases such as Dengue fever (Huang et al., 2016) and measle virus infection in susceptible mammals (Raynaud-Messina, Verollet, & Maridonneau-Parini, 2019; Sundaram, Sambandam, Shanmugarajan, Rao, & Reddy, 2017).

8.2 Evaluation of the results in light of existing knowledge and identified gaps for future study

Paper I revealed the involvement of costal fractures in the development of RFC and MFC, giving important information on a causative factor that should be mitigated, if possible, during production. Norwegian salmonid production is highly intensified, and production procedures are developed to accommodate the rapid, large-scale production. With the large quantities of fish produced, individual care is not possible, nor within the scope; however, fish health and welfare should be sustained on a population level with accommodating laws and regulations. The finding of costal fractures associated with RFC and MFC highlights a possible production-related cause that, if addressed and corrected, could reduce the prevalence of RFC and MFC. Alternatively, the costal fractures may result from factors other than direct trauma, such as poor bone health during osteopathological conditions. Although not associated with RFC and MFC, costal variations found in paper I, such as radiolucent ribs with large cavities in the cortical bone, could negatively affect the bone's strength and resistance. Increased resorption of bone has been correlated with increased fracture risk in mammals. In salmonids, increased bone and cartilage resorption has been reported in salmon suffering from phosphorus deficiency (Roberts, Hardy, & Sugiura, 2001) and during bacterial infections (Ostland, McGrogan, & Ferguson, 1997). Thus, costal fractures could potentially

increase during nutritional and disease-related pathologies. However, as seen in birds, resorption resulting in an increased diameter of the marrow cavity in bones is correlated with increased stiffness of the bone, and it is thus more likely that the observed resorption of bone in the salmon ribs is due to a life in aquatic water without the impact of gravity increasing the bone thickness. Additionally, resorption and remodeling are continuous processes in bone, in particular in fish with indeterminate growth, such as salmonids, and are an important part of developing secondary sexual characteristics such as the kype in male salmon (Perry et al., 2019; Witten & Hall, 2002). It should be noted that a local inflammation in the adjacent soft tissue could affect the resorption activity in the bone (Epsley et al., 2021). Thus, an alternative theory behind the fractured ribs is the negative effect on bone tissue due to MFC, as chronic inflammation may impair normal osteoclast and osteoblast function.

The overall occurrence of costal abnormalities in farmed Atlantic salmon in Norway has not been previously investigated. In our work, we obtained insight into this matter by conducting radiographic investigations on the control samples retrieved from commercial facilities. However, for future research, to gain a more comprehensive representation of the prevalence of costal fracture in farmed Atlantic salmon and to reveal possible causes behind these pathological changes, a larger sample size should be obtained from different producers without the selection criteria of RFC and MFC. The same approach should be taken for farmed rainbow trout, as the prevalence of costal abnormalities in the Norwegian rainbow trout production remains known. Additionally, the prevalence of costal fractures and abnormalities in wild Atlantic salmon was not determined in paper I, as the sample size of wild fish was limited, and radiographic investigations were only performed on half of the wild-caught fish. Improved knowledge on the prevalence of costal fractures in farmed and wild Atlantic salmon and rainbow trout could give important information on how to avoid these pathologies and thus reduce the development of RFC and MFC.

Describing melanization with different localization and distribution occurring in the same species brings additional knowledge to the melanization process in skeletal muscle. The abundance of melano-macrophages seen in paper II is not typically seen in red skeletal muscle inflammation induced by PRV-1 or SAV infection. This may be an atypical presentation of the conditions, or it may be a result of co-infection or due to other unknown factors. The samples were collected at slaughter, which resonates with the changes showing signs of late-stage chronic inflammation with fibrosis and regeneration. Also, the red skeletal muscle may initiate a different response compared to the white skeletal muscle. The differing physiological properties in the red (slow) and white (fast) skeletal muscle may explain a dissimilar response. In mammals, muscular dystrophies have been a source of research for several years. Although the role of fiber type differences has not been fully explained, reports have shown that fast muscle fibers are more prone to damage during contraction in DMD mice (Moens, Baatsen, & Marechal, 1993). As for viral defense mechanisms, in contrast to skeletal muscle, the host defense against PRV-1 in heart muscle in Atlantic salmon showed an early abundance of M2 macrophages scattered in the tissue without the intracellular presence of PRV-1 (Malik, Nyman, Wessel, Dahle, & Rimstad, 2021), combined with a higher regenerative rate of the tissue (Dhamotharan et al., 2020). These results illustrate the heart muscle's ability to regenerate, possibly due to the resident immune cells in the tissue (Poss, Wilson, & Keating, 2002; Nakamura & Shimozawa, 1994). As for white skeletal muscle, M2 polarized macrophages were associated with PRV-1 infection in severe MFC (Malik et al., 2021a). Here, the presence of intracellular melanin coincided with the M2 polarization, indicating that the recruited macrophages entered the tissue as non-pigmented cells that progressed to produce melanin (Malik et al., 2021a). Thus, the infected melano-macrophages were suggested to have anti-inflammatory polarization. The finding of excessive melano-macrophage presence in melanized red skeletal muscle in paper II could indicate

that the regenerative capacity in skeletal muscle is lower than in the heart, and a persistent viral infection could impede the regeneration process, as seen in MFC in white skeletal muscle. Although RT-qPCR detected virus in the red skeletal muscle, the localization of PRV-1 could not be detected in our material by ISH due to sub-optimal fixation. Additionally, although no organized granulomas were observed in paper II, only six samples with observable macroscopic changes in the red skeletal muscle were examined. Thus, the number of samples was limited, and granuloma formation in red muscle cannot be ruled out based on this investigation. For future investigations, ultra-structural cell studies focusing on melanin production in salmonid macrophages infected with PRV-1 could give interesting information on the function of melanin in these cells during infection.

In paper III, the exclusion of genetic divergencies among different Atlantic salmon origins as a causative factor has provided novel insight into the occurrence of RFC and MFC, narrowing down the search for root causes of the conditions. Muscular dystrophies in mammals are often linked to genetic diseases, most commonly being DMD, caused by dysfunction of the important sarcolemma-associated protein dystrophin, resulting in impaired muscle strength and function (Duan, Goemans, Takeda, Mercuri, & Aartsma-Rus, 2023). Due to calcium influx and leakage of DAMPs from the damaged myocytes caused by muscle contraction, the myocytes undergo necrosis, and an inflammatory reaction is initiated (De Paepe & De Bleecker, 2013). Thus, muscular dystrophies are characterized by degeneration of myocytes, necrosis, and muscle regeneration with infiltration of fibrosis and adipose tissue (Dubuisson et al., 2022; Gaspar, Vasishta, & Radotra, 2019). These similarities to MFC give sufficient grounds for suggesting heritability to be a causative factor for these changes in farmed salmonids. However, the results from paper III indicate that the causative factor for RFC and MFC lies within the production procedures, implying the importance of optimized rearing and handling conditions in Atlantic salmon and rainbow trout production. Several external factors have been suggested to induce RFC and MFC in salmonid production, and as the condition appears to be multifactorial, several aspects of the production procedures should be analyzed. The results in paper I point towards one of these contributing factors. As shown in earlier reports, PRV-1 is an important driver of inflammation in MFC in Atlantic salmon, but the viral infection is not the initial cause of RFC. Interestingly, RFC and MFC were not detected in vaccinated, PRV-1-positive fish kept in in-house tanks (Bjørgen et al., 2015). These fish were not subjected to mechanical delousing procedures, implying that the RFC and MFC detected in seawater cages in the same study were caused by dissimilar fish handling compared to in-house tanks. Organically farmed salmon are also reported to have a lower prevalence of RFC and MFC. Here, specific legislations apply for the production procedures, including water quality, oxygen levels, a higher content of FO in the feeds, the use of cleaner fish as the preferred method for sea lice treatment, and a lower maximum fish density in freshwater (20 kg/m^3) and seawater (10 kg/m^3) compared to conventional salmon production (Lovdata, 2015). All these external factor differences may contribute to the dissimilar prevalence of focal changes in the skeletal muscle in their respective production forms. However, in paper III, the maximum fish density was substantially lower than what is accepted as a max kg/m^3 in both organic and conventional production, and the fish were handled with minimal traumatic impact during lice removal. Despite these differences from conventional production, a similar prevalence of RFC and MFC were observed in paper III. Nevertheless, an important difference from organic salmon production in paper III was the use of conventional feeds, i.e., with a lower level of nutritional FO.

Although reared in fairly similar matters as conventional Atlantic salmon, direct comparative conclusions cannot be made in rainbow trout production unless the fish are kept in identical facilities. Rainbow trout acquire a higher water temperature during hatching (Noble et al., 2020) and are more prone to smoltification than Atlantic salmon. Notably, according to industry representatives, rainbow trout is a more physically active species, indicating a higher demand for

feed and oxygen. The lower prevalence of RFC in paper IV could suggest that the initial factor causing the acute inflammation is less prevalent in rainbow trout production. A common-garden experiment including both species kept in typical salmon production conditions could reveal if the difference in prevalence between the two species seen in paper IV is indeed due to genetic differences. Another reason for the discrepancy in MFC prevalence in farmed rainbow trout and Atlantic salmon could be the differing body shape between the species causing different impacts of a possible inflicted trauma. Rainbow trout are commonly shorter relative to weight compared to Atlantic salmon (Berge et al., 2023). Interestingly, the higher activity level of the rainbow trout would imply a higher impact on physical barriers in the production facilities. Thus, one could expect a higher prevalence of trauma-induced damage to the musculoskeletal system in farmed rainbow trout. However, this has not been reported, nor is this in correlation with the prevalence of RFC and MFC in paper IV. It is, however, due to the result in paper IV, likely that the difference in prevalence of RFC and MFC in the two salmonid species is not caused by different impacts of rearing procedures but rather different responses to the harmful impact.

In paper IV, several similarities were observed regarding the macroscopic and histological characteristics of RFC and MFC when comparing Atlantic salmon and rainbow trout. However, some significant differences were noted, such as a lower prevalence of the macroscopic changes and the absence of organized granulomas in histological investigations. Additionally, there was an excessive presence of small, regenerating myocytes with abundant adjacent connective tissue. The latter observation was also seen in the diffuse melanization of red skeletal muscle in paper II. However, it is worth noting that white skeletal muscle in rainbow trout had a lower presence of melano-macrophages than red and white skeletal muscle in Atlantic salmon. Thus, the shared localization of these changes suggests that the initial causative factors of RFC and MFC in Atlantic salmon and rainbow trout are likely the same, but that there are variations in the rate and process of repair and regeneration. In mammals, inflammatory mediators produced from polarized macrophages regulate the survival of FAPs, with M2 macrophages promoting the production of fibrosis and adipose tissue (Lemos et al., 2015). M2 macrophages prolong the effect of FAPs through TGB-β1 by triggering FAP differentiation. Two isoforms of TGF-β1 (TGF- β1a and TGF-β1b) have been detected in salmonids (Hardie et al., 1998; Maehr et al., 2013) and has been reported to have similar effects in fish as in mammals in terms of immune reactions. Of the two TGF-β1, particularly TGF-β1b appears to be linked to macrophages (Maehr et al., 2013). Fibrosis and infiltration of adipocytes are common findings in MFC in Atlantic salmon (Bjørgen et al., 2019; Larsen et al., 2012) and were indeed found in rainbow trout in paper IV. During muscle damage in rainbow trout inflicted by scalpel incision, TGF-β1 was upregulated, coinciding with the increase of M2 macrophages in the tissue, likely explaining the abundance of fibrosis (Schmidt, Andersen, Ersbøll, & Nielsen, 2016). The slow regenerative capacity in the skeletal muscle in Atlantic salmon MFC are further suspected to result in the unsuccessful healing of the chronic inflammation due to an ongoing M2 polarization following persistently infected macrophages. This was emphasized by the findings in paper IV, as virus was not present in the lesions, and the lesions were less prevalent. As the localization of the investigated viruses was not related to the lesions or melano-macrophages, this indicates that the low prevalence of granulomas and the lower prevalence of MFC in rainbow trout is due to a more efficient immune response with possibly a more rapid clearance of the virus. As for the regeneration process in skeletal muscle per se in rainbow trout versus Atlantic salmon, comparative studies inflicting tissue damage with identical sources should be conducted without the use of infectious pathogens, as the pathogen species-specificities and host response result in differing results of healing.

Melanization of skeletal muscle in fish has been described in many species, including salmonids, and appears to have several explanations apart from viral infections, such as parasite infection in

Portuguese pouting (*Trisopterus luscus*) (Esteves et al., 2009) and elevated dietary zinc levels in sand flathead (*Platycephalus bassensis*) (Ooi et al., 2022). Melanization of blood vessels has also been reported in both farmed (Cooper, Olsen, Seliussen, & Gannefors, 2011) and wild fish (Ooi, Ware, Lewis, Lyle, & Nowak, 2019). Although rare, peritoneal melanization in humans has been reported in association with pathological conditions in the abdominal cavity, predominantly seen with ovarian lesions in females (Kim, Suh, Song, & Ahn, 2002). A common feature for all these cases is the presence of harmful factors, such as infectious agents, cancerous development, or heavy metals. However, melanization is not consequently linked to inflammation or apparent infiltration of macrophages in the local tissue, as seen in melanized sand flat head skeletal muscle (Stocker, Haddy, Lyle, & Nowak, 2020). The melanization of the alimentary tract seen in several teleost species has not been linked to a specific cause (Fishelson, Golani, Russell, Galil, & Goren, 2012). The tissue melanization in some of the mentioned reports has thus been attributed to the presence of melanocytes and not melano-macrophages (Cooper & Midling, 2007; Fishelson et al., 2012). In mammals, melanogenesis is especially induced by nitric oxide (NO) (Lassalle et al., 2003), and the unusual location of melanocytes in the peritoneum of humans during peritoneal melanosis has been suggested to result from migration of neural crest-derived cells differentiating into melanocytes (Drachenberg & Papadimitriou, 1990; Le Douarin, Creuzet, Couly, & Dupin, 2004). Noteworthy, melanin synthesis has been reported in adipose tissue of obese humans, suggested to be a result of increased ROS in the tissue (Randhawa et al., 2009). The presence of such non-classical melanocytes may also be the case in fish, although not yet completely understood (Adameyko & Lallemend, 2010). Nevertheless, the presence of melanin-producing melano-macrophages has been shown in MFC in Atlantic salmon (Larsen et al., 2012), due to the distribution of pigment in the characteristic dendritic cells, combined with the results from Thorsen et al. (2006) and Haugarvoll et al. (2008) showing tyrosinase activity and melanin production in an Atlantic salmon leukocyte population. In this thesis, the findings of melano-macrophages in adipose tissue were recurring in all four papers. Additionally, the histological examination of the skeletal muscle in freshwater smolts in paper III revealed no pathological changes, except for melano-macrophages in the adipose tissue. The presence of melano-macrophages in relation to adipocytes could be part of an ongoing recruitment of non-pigmented macrophages from the circulation with the production of melanin at the site in response to inflammatory stimuli. Alternatively, the observed melano-macrophages may have developed from tissue-resident non-pigmented macrophages. In amphibians, macrophages were detected in adipose tissue before bone marrow establishment, but also in *Ambystoma mexicanum*, an amphibian lacking bone marrow hematopoiesis, like teleosts (Hassnain Waqas et al., 2017). Noteworthy, earlier reports have also shown other similarities between amphibians and fish, such as the presence of melanin-producing macrophages in the liver (Sichel et al., 1997), suggesting a possible evolutionary link in the development of these immune cells. In the visceral adipose tissue of rainbow trout, cells expressing MHC-II molecules have been identified, suggesting macrophages to be present (Pignatelli et al., 2014), however, the presence of melano-macrophages was not determined. Thus, the presence and role of tissue-resident macrophages in adipose tissue in rainbow trout and Atlantic salmon is somewhat unclear and should be further investigated.

As melano-macrophages are occasionally seen in non-immunological tissues and organs in response to chronic inflammation, the findings of melano-macrophages in adipose tissue could indicate a low-scale inflammation in the affected area. In chronic inflammation in skeletal muscle, FAP activation causes an increased number of adipocytes in the affected tissue. Thus, an underlying cause of steatitis in the fish could further increase the local melano-macrophage infiltration in the inflamed skeletal muscle. In salmonid production, increased nutritional level of LC-PUFA, herein the anti-inflammatory EPA, and DHA (Wu, Ting, & Chen, 2003), has shown to reduce the prevalence of MFC in

the fillet (Lutfi et al., 2022; Sissener et al., 2016), and even the severity of the pathological lesions associated with HSMI (Martinez-Rubio et al., 2012). The effect of PUFA levels on MFC could also be the reason for the difference in prevalence in conventionally farmed versus organically farmed salmon, with an important productional difference being the content of marine fish oils in the diet (Esaiassen et al., 2022). In general, PUFA is necessary for adequate function of the immune system (Kiron et al., 1995), however, a higher level of PUFAs will bring increased fatty acid peroxidation in the tissue (Stéphan, Guillaume, & Lamour, 1995) and a maximum dietary level of PUFA has been suggested (Olsen, Loevaas, & Lie, 1999). In the study conducted by Twibell et al. (2017), steatitis in rainbow trout was associated with a high level of FO in the diet. Interestingly, although increased VO in exchange for the beneficial FO in commercial feeds has been shown to result in both reduced fish health and increased prevalence of MFC (Bou et al., 2017; Sissener et al., 2016), a substitution of FO with canola oil reduced the severity of steatitis in rainbow trout (Twibell et al., 2017), and importantly, the prevalence of MFC in slaughter sized fish, (Hatlen et al., 2022), without having adverse effects on other fish health parameters (Ruyter et al., 2022; Ruyter et al., 2019). These results indicate that canola oil is an appropriate replacement of FO in the diet of salmonids regarding melanization of the skeletal muscle and adipose tissue. As melano-macrophages are present in both the adipose tissue and skeletal muscle in MFC and the reduction of inflammatory conditions in these tissues are associated with the same dietary source, the link between steatitis and MFC should be further investigated.

9 CONCLUSIONS

This thesis aimed to reveal factors causing red and melanized focal changes (RFC and MFC) in Norwegian-farmed Atlantic salmon and rainbow trout. To investigate possible causes, several hypotheses were tested through observations and investigations on representative samples in papers I-IV.

Paper I: The hypothesis that red and melanized focal changes are associated with pathological conditions in the ribs was accepted. This hypothesis was postulated as RFC and MFC are predominantly found in the cranioventral part of the abdominal wall, indicating the causative factor to be related to the specific localization. The ribs in salmonids are closely related to the abdominal skeletal muscle, and pathological changes in the ribs are suggested to cause local inflammation in the adjacent soft tissue. Paper I confirmed the association between costal fractures and RFC/MFC. This was supported by the revealed anatomical properties of the ribs, indicating that a costal fracture could lead to a hemorrhage and acute inflammation in the local tissue, followed by healing of the fracture resulting in callus formations. Callus formations were only associated with MFC, not RFC, coinciding with the association of acute costal damage and acute inflammation (RFC) and the late phase of bone healing and chronic inflammation (MFC). However, RFC and MFC were also observed with no costal fractures, indicating that other unknown factors also can cause local inflammation in the skeletal muscle.

Paper II: The hypothesis that melanized changes in skeletal muscle are similar in both red and white skeletal muscle was partly accepted. As diffuse melanization of red skeletal muscle differs from MFC in both distribution in the tissue and prevalence, this hypothesis was postulated. Red skeletal muscle differs from white skeletal muscle in terms of vascularization and metabolism, thus, the pathogenesis behind the melanization may vary from white skeletal muscle. However, due to the different location and distribution in the tissue, the initial cause may also differ from that causing RFC/MFC. In paper II, melanization was seen with a clear demarcation zone separating the red and non-affected white skeletal muscles. The microscopic changes were similar to those found in MFC; extensive fibrosis was present between necrotic and regenerative myocytes, and an abundance of melano-macrophages was seen interspersed in the lesioned areas, indicating diffuse granulomatous inflammation. However, no organized granulomas were observed. This could be due to the limited sample size or the different tissue healing and pathogen elimination processes in the red skeletal muscle. Although PRV-1 is suggested to be an important driving force of chronic inflammation in MFC, the virus is not suspected to be the initiating cause of the focal changes. A characteristic pathological finding in Atlantic salmon's heart and skeletal muscle inflammation (HSMI) is skeletal muscle inflammation affecting primarily the red skeletal muscle. In this study, the sampled fish were diagnosed with HSMI during the seawater phase of production; thus, combined with a statistically significant lower Ct value for PRV-1 in the affected samples, the results indicate that the observed changes could be caused by an outbreak of HSMI, with a persistent infection of PRV-1 leaving a non-resolved chronic inflammation as seen in MFC.

Paper III: The hypothesis that the prevalence and development of melanized changes are influenced by the genetic differences between farmed and wild Atlantic salmon was rejected. This hypothesis was formed as no observations of RFC and MFC have been reported in wild Atlantic salmon. This leads to the question of whether the relatively high prevalence of the changes in farmed Atlantic salmon is due to the external rearing conditions or the genetic divergence between wild and farmed salmon, a consequence of meticulous selective breeding during the last decades. To uncover this,

Atlantic salmon of farmed, wild, and hybrid origin were kept in identical facilities from hatching to slaughter in a so-called common garden experiment. The focal changes were observed only after seawater transfer, and the general prevalence of RFC and MFC coincided with earlier reports, with 1-5% RFC throughout the seawater phase and an increasing occurrence of MFC, with 25-30 % at slaughter. There were no statistical differences in prevalence between the different origin groups. Additionally, histological examination revealed no origin differences in terms of inflammatory responses. These results indicate that the contrasting presence of RFC and MFC in farmed versus wild Atlantic salmon is due to external factors in the salmonid production, such as feeding, handling, and other production procedures, and not due to genetic differences between farmed salmon and its wild counterparts.

Paper IV: The hypothesis that the prevalence and morphological characteristics of melanized focal changes in farmed rainbow trout are similar to that in Atlantic salmon was rejected. In farmed rainbow trout production, RFC and MFC observations are less frequent than in Atlantic salmon production, and thus this hypothesis was formed. As revealed in paper III, the dissimilar frequency of changes could be due to external production procedures. However, the Norwegian production of rainbow trout is similar to that of Atlantic salmon, suggesting that internal (genetic) differences between the two species are of greater importance to the dissimilar prevalence. Macroscopically, rainbow trout MFC were predominantly seen in the cranioventral area of the abdominal wall, equivalent to the most common location in Atlantic salmon. In contrast, RFC were only observed in one of 1293 fish, and the maximum prevalence of MFC was 6,47%. In histological examinations, chronic inflammation with abundant fibrosis was detected. The presence of melano-macrophages was lesser than compared to Atlantic salmon MFC, and organized granulomas were only detected in one fish. PRV-1 and SAV were detected in the skeletal muscle through ISH, but not in relation to the lesions. This contrasts with Atlantic salmon MFC, where PRV-1 is observed in relation to severe lesions and granulomas and thus is likely to play an important role in chronic, non-resolved inflammation. PRV-3, the virus causing HSMI in rainbow trout, was only detected in heart tissue, not skeletal muscle. The location of the changes in the abdominal wall implies that the initial causes of RFC in farmed rainbow trout are the same as in farmed salmon, possibly with a lower impact resulting in a lower prevalence. However, the inflammatory reaction and viral impact in MFC differed between the two species, suggesting that genetic divergencies affecting these responses are of significance for the duration and healing of the tissue, consequently resulting in faster healing and, thus, a lower prevalence of MFC in farmed rainbow trout.

In sum, this thesis shows that Atlantic salmon RFC, and consequently MFC, are likely caused by external factors found in the production procedures in Norwegian salmonid aquaculture, and not by genetic differences within the species. One of these external factors may cause costal fractures, leading to RFC and later MFC development. However, as RFC and MFC were also observed without costal abnormalities, further research is required to reveal additional external causative factors. The atypic distribution of melanization in red skeletal muscle suggests a different initial causative factor than for RFC and MFC; however, the duration and severity of the lesion could point to persistent infection of PRV-1, as seen in MFC. The unlike prevalence of RFC and MFC in farmed rainbow trout versus Atlantic salmon, combined with the dissimilar distribution of PRV-1 in rainbow trout skeletal muscle, underline previous reports on PRV-1 being an important driving force in the development of chronic inflammation in Atlantic salmon MFC.

10 FUTURE PERSPECTIVES

This thesis has contributed valuable insight into the origins and development of RFC and MFC. Paper I and III results indicate the importance of controlling external factors in salmonids production. These procedures and milieu factors encompass a wide range, including water temperature, timing of smoltification, timing of sea transfer, nutritional content, hardness of the pellets, handling during transportation, vaccination, medical/mechanical treatments, and general stressors causing unease in the population. To identify single causative factors, focusing on production facilities with lower prevalence of RFC and MFC would be warranted. Previous research has revealed lower prevalence in organic Atlantic salmon production and land-based production. Also, as demonstrated in Paper IV, while the inflammatory response differs in rainbow trout, the prevalence of RFC in rainbow trout is lower than in Atlantic salmon. Thus, the initiating factor, or the production-related factor responsible for these changes, could be less prevalent in rainbow trout production. For instance, as implied by the results in paper I, if lower rates of costal fractures are observed in these rearing facilities, this could potentially uncover the specific procedures causing such fractures. Subsequently, beneficial changes could be implemented into conventional Atlantic salmon production. Hence, it is important to conduct comparative investigations on handling and treatment in various production facilities.

As a result of cost-efficiency-driven adjustments, the replacement of fish oils with vegetable oils has been linked to an increased prevalence of melanized changes, primarily due to reduction of the important anti-inflammatory omega-3 fatty acids EPA and DHA. However, maintaining a high level of fish oils may also have adverse effects on the inflammatory state within the adipose tissue, as seen in farmed fish with pansteatitis. Research conducted thus far on specific lipid sources, such as canola oil, suggest that some vegetable oils can yield positive effect not only on adipose tissue but also on skeletal muscle, leading to a lower prevalence of MFC when compared to standardized fish feeds. Given that salmonid skeletal muscle comprises a significant portion of fatty tissue, and adipocyte infiltration frequently occurs within chronic inflammatory lesions, it is important to investigate any potential correlation between steatitis and MFC. An increase in inflammation within the fillet may result in increased presence of anti-inflammatory melano-macrophages, as shown in previous reports on macrophage polarization in MFC. Furthermore, it is not only the lipid source itself, but also the nutritional fat content in total that could contribute to obesity-related health issues as seen in humans. The combination of fast growth in farmed salmonids and excessive lipids in their feed could potentially serve as a source of inflammation in the skeletal muscle-related adipose tissue. Thus, low-cost feeds containing selected vegetable oils without compromising the fish robustness and increasing the inflammatory state are warranted. Further research on the consequence of alternative lipid sources affecting inflammatory conditions, seen in light of MFC, could give valuable knowledge to further understand the effect of feeds on this condition.

This thesis supports the role of PRV-1 infection in driving the development of MFC in farmed Atlantic salmon, particularly when contrasted with the results in rainbow trout, where the virus was not localized in relation to lesioned areas. Given that PRV-1 infection is inevitable in farmed salmon in Norway, increased host defence against the infection is desirable. Vaccination has successfully protected against several bacterial infections in salmonid farming and is a far more preferrable approach compared to medical treatments. However, as PRV cannot be cultivated in vitro, a lot is still unknown about the virus entry-mechanism and the organism's ability to eliminate the virus. These gaps make the development of efficient vaccines a complex task. In alignment with previous reports, the results in paper IV implies that rainbow trout have dissimilar response to PRV-1 infection compared to Atlantic salmon, although both PRV-1 and PRV-3 may infect both species. Due to cross-

protection against PRV-1 in PRV-3 infected salmon, an alternative approach using live PRV-3 vaccines has been suggested, however several challenges are related to this type of immunization, such as the possible infection risk of rainbow trout and of wild salmon. Alternatively, the genetic variations causing the dissimilar reactions in response to PRV-1 and PRV-3 infection could be further investigated in comparative studies between Atlantic salmon and rainbow trout, as has been conducted during selective breeding with genomic selection in Atlantic salmon against several infectious agents.

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12 ENCLOSED PAPERS I-IV

I

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ORIGINAL ARTICLE

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Anatomical and pathological characteristics of ribs in the Atlantic salmon (*Salmo salar* L.) and its relevance to soft tissue changes

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Abstract

Studies on the anatomical and pathological characteristics of ribs in farmed Atlantic salmon (*Salmo salar* L.) are warranted due to their possible association with red and melanized focal changes (RFC and MFC) in the fillet, a major quality and animal welfare concern. In this work, we provide an anatomical description of ribs based on radiographical and histological analyses. We also address various pathological rib changes and their association to RFC and MFC. In total, 129 fish were investigated; captured wild ($n = 10$) and hatchery reared ($n = 119$) Atlantic salmon (3.5–6.1 kg). The fish were selected based on the macroscopic presence of RFC, MFC or no changes (controls). Radiographic results revealed costal abnormalities in all fish groups. By histological investigations of the variations herein, our results provide new insight into the anatomical characteristics including vascularization within the ribs; a potential site for haemorrhage following costal fractures. Costal fractures were detected by radiology in 40 of 129 samples (RFC: 38.4%, MFC: 47.2%, controls: 9.5 %). A statistically significant association was found between costal fractures and red ($p = 0.007$) and melanized changes ($p = 0.000$). However, red and melanized changes were also observed in samples with no costal fractures ($n = 45$), indicating that also other factors influence the development of RFC/MFC.

KEYWORDS
Atlantic salmon, fillet, histology, melanin, radiology, ribs

1 | INTRODUCTION

Teleost bones show many similar morphological traits to mammalian bones. Both mammalian and teleost bone collars are covered by periosteum, and cortical bone is oriented in longitudinal lamellas surrounding a medullar cavity lined with endosteum (Cohen et al., 2012; Davesne et al., 2019; Jiao et al., 2020; Moss, 1961, 1962; Weiss & Watabe, 1979; Witten & Huysseune, 2009). Salmonids are regarded as primitive teleosts (Davesne et al., 2019), in which a characteristic trait is the presence of osteocytes embedded in the lamellar bone classified as cellular bone, as found in mammals (Moss, 1961;Moss, 1963). Advanced teleost species such as tilapia

(*Oreochromis niloticus*) and medaka (*Oryzias latipes*) are classified with acellular bone, that is, bone lacking embedded osteocytes (Boglione et al., 2013; Cohen et al., 2012; Kölliker, 1857; Moss, 1961). Bone formation is performed through perichondral, endochondral or intramembranous ossification and in large teleosts such as the Atlantic salmon (*Salmo salar*), the presence of spongious bone has been described (Moss, 1961, 1963; Weigele & Franz-Odendaal, 2016; Witten et al., 2000; Witten & Hall, 2002). In contrast to mammals, teleosts have no functional bone marrow in the medullar cavity of the bone (reviewed by Bjørgen & Koppang, 2021). The medullar cavity consists of either chondrocytes or, after resorption of cartilage by chondroclasts, adipose tissue (Boglione et al., 2013; Jiao et al., 2020; Moss, 1963; Weigele & Franz-Odendaal, 2016; Willett et al., 1999; Witten et al., 2001; Witten & Huysseune, 2009).

In teleost fish, the ribs (*costae*) are part of the axial skeleton, extending from the vertebral bodies in a ventrolateral direction into the sub-peritoneal fascia on both sides of the abdominal cavity (Kryvi & Poppe, 2016; Roberts, 2012). Investigations done on silver carp (*Hypophthalmichthys molitrix*) have shown that the ribs develop by perichondral ossification with an elongation process through type II endochondral ossification, with no bone tissue formed in the medullar cavity (Soliman, 2018). Together with the thick abdominal musculature and covered by parietal peritoneum, the ribs function as a protective shield for the abdominal organs. They also act as anchors for muscle attachment, thus contributing to the swimming movement of the fish. Although the structure of ribs in fish, including Atlantic salmon, has been described in previous reports (Horton & Summers, 2009; Jiao et al., 2020; Jiménez-Guerrero et al., 2022; Kague et al., 2019; Nie et al., 2017; Patterson & Johnson, 1995; Roberts, 2012; Soliman, 2018), studies on the anatomy of ribs in farmed Atlantic salmon with a broad histological approach are scarce. In farmed salmon and other reared fish species, pathological changes and deformities in bones are not uncommon and have been associated with several production-related factors (Aunsmo et al., 2008; Baeverfjord et al., 1998; Boglione et al., 2014; Eriksen et al., 2006; Gil Martens et al., 2010; Gislason et al., 2010; Martini et al., 2021). Insufficient diet giving phosphorus, ascorbic acid and vitamin D deficiency has been proved to affect bone development and bone weakness in both mammals and teleosts (Baeverfjord et al., 1998; Darias et al., 2011; Roberts et al., 2001). In salmonids, vertebral deformities have been reported as the most frequent skeletal abnormality, with several different malformations in the vertebral column such as lordosis, kyphosis and scoliosis, vertebral fusions and cross-stich vertebra (Fjelldal et al., 2012; Trangerud et al., 2020; Witten et al., 2009). Thus, most studies have focused on changes in the vertebra, and not the ribs.

Costal changes seem especially interesting in relation to the condition termed melanized focal changes (MFC), which is a major quality concern in the fillet of farmed salmon. Such changes are typically restricted to a focal area in the cranio-ventral part of the fillet (Bjørgen et al., 2019), with affected musculature reaching medially from underneath the peritoneum and continuing towards the red musculature laterally. Based on primarily radiological analysis, an association between costal abnormalities and MFC was reported by Jiménez-Guerrero et al. (2022). However, MFC were also found in fish without costal abnormalities, and environmental factors were suggested to be determinant for the development of MFC (Jiménez-Guerrero et al., 2022). The average prevalence of MFC at slaughter is about 20% (Mørkøre et al., 2015), and their occurrence is a reason for downgrading or cassation, leading to substantial economic losses. In the early stage of the condition, a focal haemorrhage occurs in the same restricted area. These changes appear red (macroscopically) and have thus been termed red focal changes (RFC). Histologically, acute necrosis and extra-vasal erythrocytes dominate within the tissue (Bjørgen et al., 2019, 2020). As the RFC develop, the tissue becomes increasingly necrotic and fibrotic, and leukocytes are recruited to the area. Among these are the melanomacrophages, a pigment-producing leukocyte responsible for the discolouration of the fillet. Instead of healing, a chronic granulomatous inflammatory condition may develop, sometimes with well-organized granulomas (Koppang et al., 2005; Larsen et al., 2012). This state of the condition has been associated with the presence of piscine orthoreovirus 1 (PRV-1) (Bjørgen et al., 2015, 2019). PRV-1 antigen has been shown to be walled off in granulomas, and local replication of PRV-1 is believed to be the driving power of this non-resolving condition (Bjørgen et al., 2020). However, RFC can occur prior to PRV-1 infection (Bjørgen et al., 2019), and their initial cause is still unknown. Their restricted focal location argues for an underlying anatomical predisposition, possibly associated with costal injury and/or costal fractures. In mammals, but also in fish, bone fractures can affect the neighbouring soft tissue and can cause haemorrhage (de Haan et al., 2016). This in turn is followed by acute inflammation (Loi et al., 2016; Marsell & Einhorn, 2011; Schindeler et al., 2008). Thus, a similar pathogenesis is possible in salmon, where haemorrhage and acute inflammation following a costal fracture may develop into a chronic, granulomatous inflammation driven by the presence of PRV-1.

The overall aim of this study was to investigate variations in costal anatomy in Atlantic salmon, herein addressing various changes in the ribs and in the neighbouring musculature, possibly leading to the development of RFC/MFC. This was approached by collecting a comprehensive material from different groups of fish including farmed, wild, and experimentally kept fish, where samples of both affected (RFC/MFC) and unaffected musculature were collected. By performing macroscopic, radiographic and histological investigations, we describe the anatomical features, the different pathological changes and the association between such changes and RFC/MFC.

2 | MATERIALS AND METHODS

2.1 | Fish origin

Fillets containing ribs were obtained from three different locations in Norway (Table 1). In total, we studied the ribs and adjacent soft tissue in relation to RFC and MFC from 129 individuals.

TABLE 1 Fish origin and key information

Population	Location	Samples (*n*)	Sea transfer (date)	Harvest (week)	Weight at slaughter (mean)	Vaccination	Diagnosis
1	Bremnes Seashore	40	21.11.2017	21/2019	4.70 kg	Aquavet PD 7 VET	PD, CMS
2	Bremnes Seashore	18	17.03.2018	26/2019	3.50 kg	Alpha Ject Micro 1 PD/6	PD, CMS, HSMI
3	Bremnes Seashore	32	08.09.2019	5/2021	5.99 kg	Alpha Ject Micro 1 PD/6	PD, CMS patraurellosis
4	Bremnes Seashore	6	21.09.2021	34/2022	4.10kg	Aquavaq 6	Pastaurellosis, mycobacteriosis, PRV[a], PMCV[a]
5	Matre Research St.	23	22.05.2018 20.08.2018	36/2019	4.39 kg	Aquavac 6	PRV[a]
6	Drammenselven	6	x	46/2019	6.13 kg	x	PRV[a]
		4	x	48/2020	5.1 kg	x	

[a]Virus detected by RT-qPCR, but diagnosis not confirmed.

A total of six different samplings were conducted (population 1–6; Table 1), where population 1–4 were collected at slaughter, originating from a commercial aquaculture facility, Bremnes Seashore, Bømlo, Norway. Population 5 included farmed, wild and farmed X wild hybrid salmon from Matre Research Station, Institute of Marine Research, Matre, Norway. The farmed salmon in this population were of MOWI origin, and the wild salmon were obtained from wild caught parent's eggs and sperm fertilized at the premises in Matre. Hybrids represented a combination of wild and farmed eggs and sperm. After hatching, wild, farmed and hybrid fish were kept under common rearing conditions, i.e., tanks and sea water cages (Debes et al., 2021). Population 6 consisted of wild spawning salmon caught in the river Drammenselven in the eastern part of Norway.

2.2 | Sample handling and macroscopic evaluation

At slaughter, the fillets were sampled based on macroscopic evaluation of the abdominal wall, that is, peritoneum, ribs and adjacent white skeletal muscle. Fillets with no appearent discolouration were sampled as controls. Macroscopic discolouration included RFC and MFC and was graded using the classification system developed by MOWI, with grades from 0 to 3 with increasing severity (Bjørgen et al., 2019). The sampling of population 1–4 was conducted by the staff at Bremnes Seashore and shipped on ice to the Norwegian School of Veterinary Science (NMBU), Adamstuen, Oslo. Here, the samples were transferred to buffered formalin for fixation. The samples in population 5 and 6 were also collected at the time of slaughter/spawning and were transferred to formalin immediately after dissection. The weight and length of each fish in these populations were registered prior to dissection. Samples from population 1–4 and 6 included the ribs in their total length, while in population 5, samples were retrieved only from the area with macroscopic discolouration and equivalent areas for controls.

Prior to radiographic investigations, the abdominal walls were palpated to reveal costal abnormalities and fractures. The size of the samples in population 1–4 and 6 were approximately 10 × 15 cm, as for population 5 the sample size was about 5 × 6 cm, with some variation. Prior to fixation in buffered formalin, all samples but for population 5 were photographed to record macroscopic information. For further investigations, the samples were grouped according to discolouration and arranged into groups; group A: No macroscopic discolouration; group B: RFC and group C: MFC.

2.3 | Radiographic investigations

Radiographic investigations were conducted at the radiology department at the NMBU premises on Adamstuen, by a direct digital system (SoundEklineSeries DR). The samples were labelled according to population and sample number and then placed on trays in order of correct numbering. The samples from Bremnes were

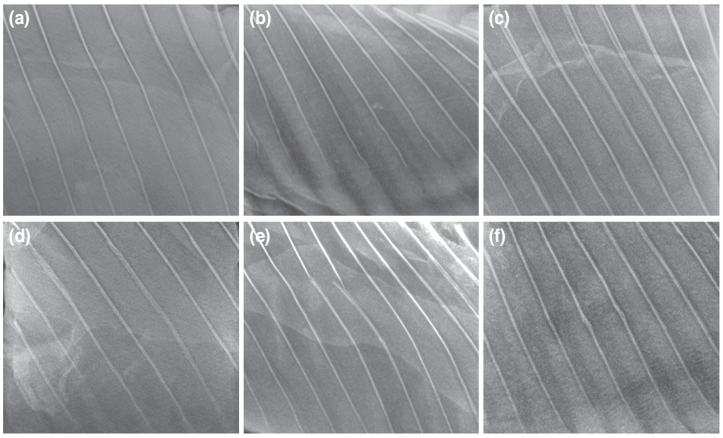

FIGURE 1 Radiological results, ribs in farmed Altantic salmon (*Salmo salar L.*) lateral aspect. (a) Proximal part of ribs showing axis deviation noted as mild bending of axis. (b) Distal part of ribs showing axis deviations noted as wave shaped ribs. (c) Proximal part of ribs with a central radiolucent medulla. (d) Mid part of ribs with focal radiolucent appearance. Distal parts of ribs are also wave shaped. (e) Mid part of rib with costal fracture. (f) Distal part of ribs with focal thickening noted as callus formations.

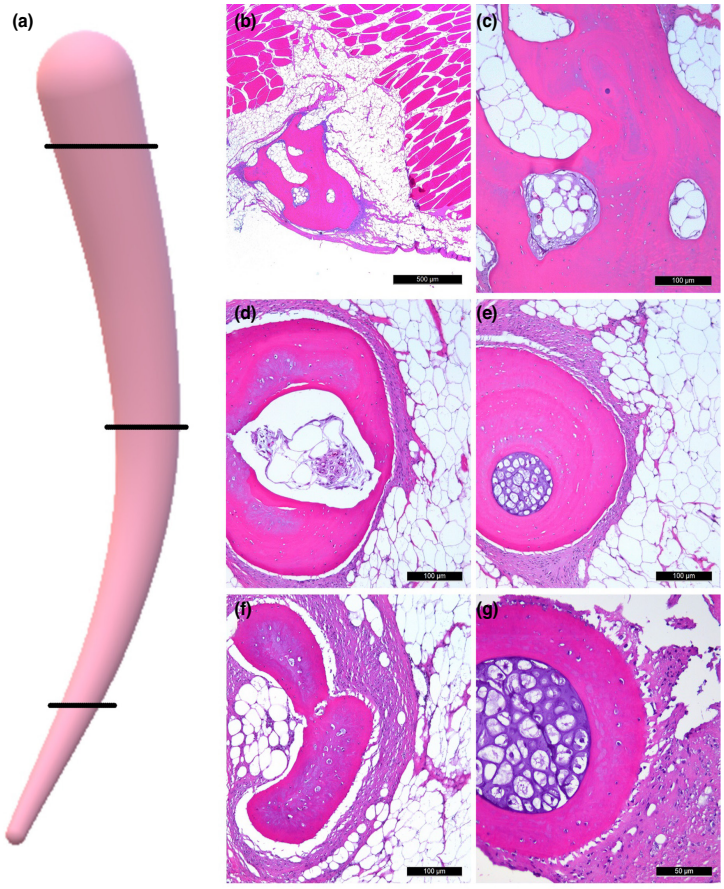

FIGURE 2 Atlantic salmon ribs. Schematic illustration of ribs (a), with cross sections showing variations of proximal (b, c), mid (d, e) and distal (f, g) parts. All sections are retrieved from the same individual (farmed salmon). Note the different scale bars in proximal and distal parts. (b) Proximal part, irregular circumference of rib with several large cavities in the cortical bone. Scale bar: 500 μm. (c) Proximal part, higher magnification of section seen in 'b'. Cavities in the cortical bone containing adipocytes. Blood vessels in the medullar cavity. Scale bar: 100 μm. (d) Mid part, round circumference. Medullar cavity with blood vessels. Scale bar: 100 μm. (e) Mid part, round circumference. Medullar cavity containing chondrocytes. Scale bar: 100 μm. (f) Distal part, irregular circumference. No apparent medullar cavity seen. Scale bar: 100 μm. (g) Distal part, round circumference. Medullar cavity containing chondrocytes. Scale bar: 50 μm.

radiographed in groups of 6, while the samples from Matre were radiographed in groups of 12, as the samples were smaller. The first six samples in population 6 were not radiologically examined, and the last four samples were radiographed in one group. After radiography, the samples were returned to buffered formalin to avoid dehydration.

2.4 | Statistical analyzes

To investigate association between costal changes and macroscopic discolouration, a chi-squared test followed by a logistic regression was conducted using STATA (StataCorp. 2019. Stata Statistical Software: Release 16: StataCorp LLC).

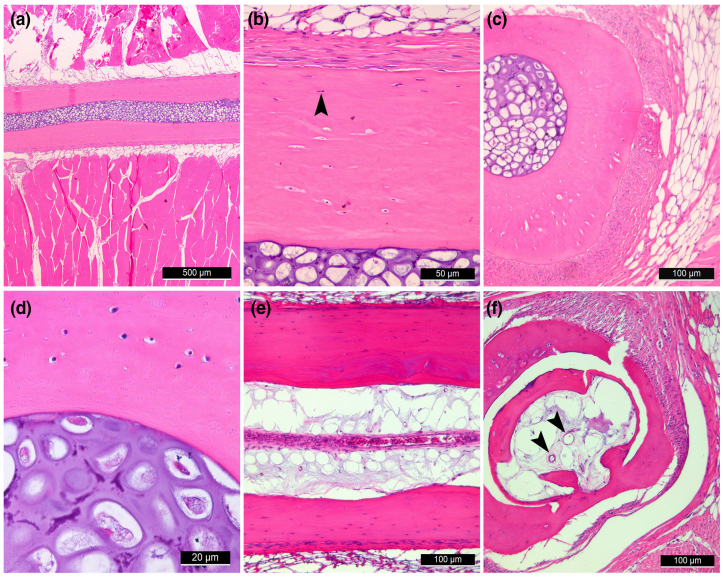

FIGURE 3 Histological investigation, group A. Anatomical characteristics of ribs and adjacent soft tissue in farmed and wild Atlantic salmon with no detected costal or adjacent soft tissue changes. All sections are retrieved from wild salmon. (a) Longitudinal section of rib. Hypertrophic chondrocytes in the medullar cavity and adjacent soft tissue, that is, adipose tissue and white skeletal muscle. Scale bar: 500 µm. (b) Longitudinal section. Osteocytes (arrowhead) embedded in the cortical bone, with parallel-oriented lamellae. Periosteum covering the bone collar. Scale bar: 50 µm. (c) Cross section. Chondrocyttic core, cortical bone and adjacent soft tissue surrounding the rib. Scale bar: 100 µm. (d) Cross section. Osteocytes embedded in bone tissue, no apparent osteons. (e) Longitudinal section. Medullar cavity with adipose tissue and a longitudinal oriented blood vessel. Scale bar: 100 µm. (f) Cross section. Medullar cavity with adipose tissue and multiple vessels seen (arrowhead). Scale bar: 100 µm.

2.5 | Histological investigations

Following fixation and radiological investigations, the samples were decalcified in 0.5 M pH 8 EDTA solution (disodium ethylenediaminetetraacetate, 2H2O) for a minimum of five days, according to size. The samples were further sectioned for block preparation to include the area containing RFC or MFC, and/or costal abnormalities revealed by radiographic investigations. Following paraffin embedding performed according to standard procedures, the slides were sectioned in 2 µm thickness and transferred to glass slides. After incubation at 37°C for 36–48 h, the sections were deparaffinized in xylene, rehydrated through alcohol baths and stained according to standard haematoxylin and eosin staining protocols.

2.6 | Pathogen detection

Farmed salmon in population 1–4 originated from larger fish groups at Bremnes Seashore, Bømlo. Registered diagnoses were detected through fish health and pathogen surveillance at the production site (Table 1). In population 5 and 6, samples from spleen were collected on RNA*later* and sent to PatoGen AS, Ålesund, Norway, for RT-qPCR-analysis accredited and validated to ISO7025 standards for the detection of piscine orthoreovirus 1 (PRV-1).

3 | RESULTS

3.1 | Macroscopic evaluation and classification of changes

Macroscopic evaluation was conducted in each population (1–6). In population 1 (Bremnes), six samples contained RFC, 29 samples contained MFC and five samples had no macroscopic discolouration. In population 2 (Bremnes), seven samples contained RFC and 11 samples contained MFC. In population 3 (Bremnes), 12 samples contained RFC, one sample contained MFC and the remaining 19 samples had no macroscopic discolouration. None of the samples in population 4 (Bremnes) showed macroscopic discolouration. In population 5 (Matre), one sample had RFC, 14 samples had MFC, and eight samples showed no macroscopic discolouration. No macroscopic discolouration was detected in population 6 (wild salmon). Changes in population 1, 2 and 3 were located mainly in the ventral area of the abdominal wall. All changes were graded 1–3. Based on macroscopic evaluation, the samples were grouped accordingly: No macroscopic discolouration (group A) $n = 48$, RFC (group B) $n = 26$, MFC (group C) $n = 55$.

In group B, seven samples were classified as grade 3, while the rest showed mild discolouration; nine samples with grade 2 and ten samples with grade 1. Mixed changes (red and melanized combined) were present in three samples, but haemorrhage dominated

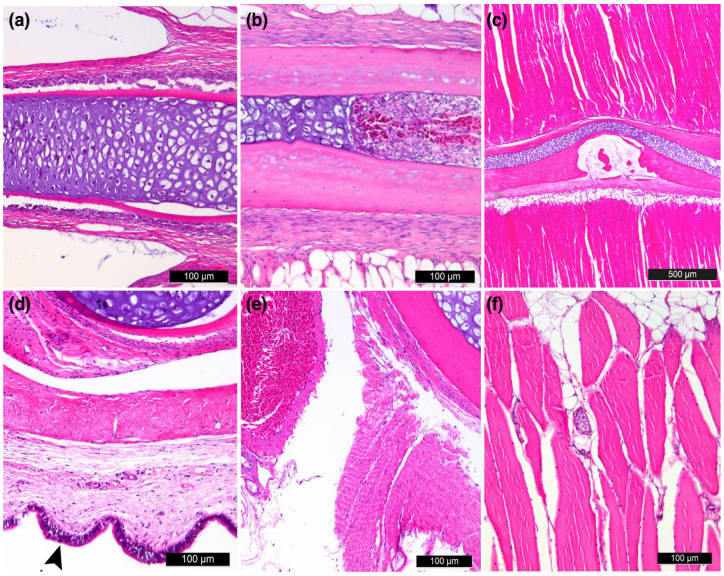

FIGURE 4 Histological investigation, group A. Anatomical characteristics of ribs and adjacent soft tissue in farmed and wild Atlantic salmon with no detected costal or adjacent soft tissue changes. (a) Farmed salmon. Distal part of rib containing a zone of proliferative chondrocytes in the medullar cavity. Hyperthrophic chondrocytes seen on the right. Scale bar: 100 µm. (b) Farmed salmon. Rib showing central medulla with abrubt demarcation in the transitional zone. The chondrocytic core is intact prior to the transitional zone. Blood vessels and cell infiltration post transition. Scale bar: 100 µm. (c) Wild salmon. Rib showing area of bone resorption in the cortical bone with an intact chondrocyttic core. Note the sparse amount of adipose tissue adjacent to the rib. Scale bar: 500 µm. (d) Wild salmon. Cross section showing rib with adjascent soft tissue facing the abdominal lumen; parietal peritoneum (arrowhead), interstitial connective tissue and elastic fibres. Scale bar: 100 µm. (e) Wild samon. Cross section of rib showing adjacent blood vessel. Scale bar: 100 µm. (f) Farmed salmon. Nerve located in adipose tissue between myocytes adjacent to rib. Scale bar: 100 µm.

the changes and were thus classified as red. In group C, five samples had grade 1, 18 samples had grade 2 and 32 samples had grade 3 MFC. Costal fractures were only detected in five samples by palpation.

3.2 | Radiographic investigations

Radiographic analysis revealed ribs with changes of different types and distribution. The results ranged from no observable changes to ribs with axis deviations, that is, mild bending of axis and distal wave shaped ribs (Figure 1a,b), radiolucent medulla in proximal to mid areas of the ribs (Figure 1c,d), single or multiple costal fractures (Figure 1e), and focal thickening with varying opacity noted as callus formations (Figure 1f). Different costal changes could be detected within the same sample. In group A (no macroscopic discolouration), 9.5% showed costal fractures. Group B (RFC) had a prevalence of 38.4% costal fractures. In group C (MFC), 47,2% of the fillets had fractured ribs. Costal fractures and callus formations were seen only in farmed salmon.

3.3 | Statistical analysis

Statistical analysis was conducted on all radiological findings in relation to macroscopic discolouration. Due to the sample sizes, population 5 was not included in statistical tests on costal changes where the entire length of the rib was required (radiolucent medulla and axis deviations). Ribs with radiolucent medulla and axis deviations were observed in all groups (A, B and C) and showed no statistically significant association with RFC/MFC (p =>0.05). In samples with costal fractures and callus formations, discolouration was often present. There was a statistical significant association between costal fractures in fillets detected by radiography, and fillets containing RFC (OR, p = 0.007) and MFC (OR, p = 0.000). There was no association between callus formations and RFC (p =>0.05); however, there was a statistical significant association between MFC and callus formations (OR, p = 0.000) found on radiographic investigations.

3.4 | Histological investigations

3.4.1 | Anatomical characteristics of Atlantic salmon ribs

The anatomical characteristics of ribs and adjacent soft tissue were characterised in group A (no macroscopic discolouration). The circumference of the rib was round or oval and smooth or irregular shaped based on the position in question (proximal, mid or distal part) (Figure 2a–g). The ribs displayed a central medullar cavity holding hypertrophic chondrocytes, with layers of a cortical bone

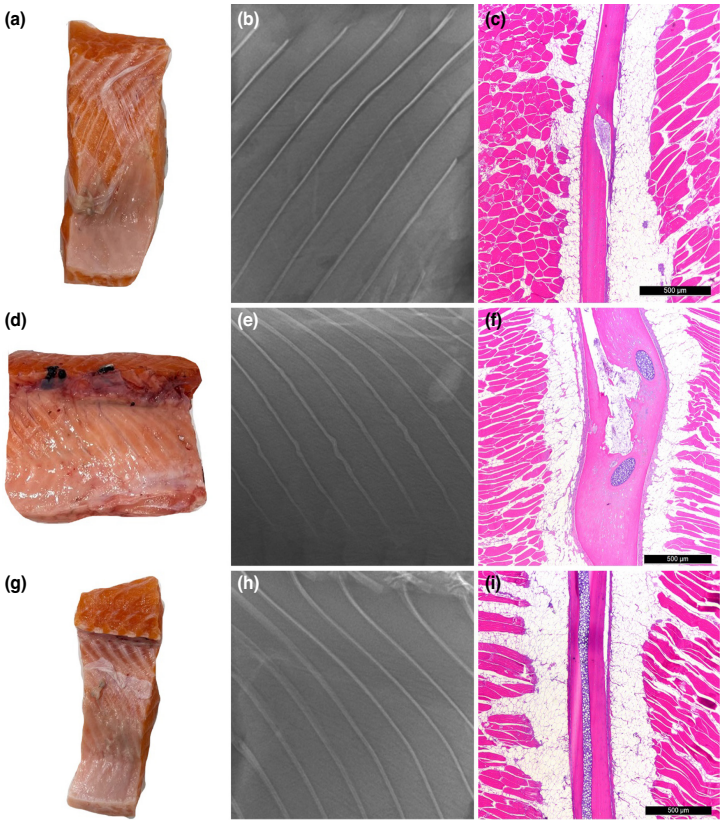

FIGURE 5 Histological investigations of rib changes observed on radiography. Mild bending of rib axis (a-c), distal wave shaped ribs (d-f) and radiolucent medulla (g-i). (a) Macroscopic details of fillet, no discolouration of soft tissue present. (b) Radiographic result of fillet showing mild bending of rib axis. (c) Histological changes of rib with mild bending of axis. Resorption cavity seen in the bone tissue. No pathological changes in surrounding soft tissue. Scale bar: 500 µm. (d) Macroscopic details of fillet, no discolouration of soft tissue present. (e) Radiographic results of fillet showing distal wave shaped ribs. (f) Histological changes of rib with wave shaped appearance. Irregular shaped rib, with the chondrocyttic medulla partly visible in the section. Resorption cavity containing blood vessel seen in a longitudinal direction in rib. No pathological changes in adjacent soft tissue. Scale bar: 500 µm. (g) Macroscopic details of fillet, no discolouration of soft tissue present. (h) Radiographic results of fillet showing ribs with radiolucent medulla. (i) Histological changes of rib with radiolucent medulla. The chondrocyttic medulla and surrounding bone tissue appears intact, no pathological changes in neighbouring soft tissue. Scale bar: 500 µm.

surrounding the centrum (Figure 3a). The bone lamellae were presented with a parallel orientation with no apparent osteons and the bone contained osteocytes embedded in bone matrix (Figure 3b). The cortical bone was covered by periosteum, connective tissue and adipose tissue, with white skeletal muscle tissue adjacent to the proximal and mid part of the ribs (Figure 3a,b). In contrast, distal parts of ribs were surrounded by abundant adipose and connective tissue. In general, wild salmon showed sparse amount of adipose tissue adjacent to the ribs compared to farmed salmon. A cross section of the ribs showed all the structures described above, with chondrocytes in the cartilaginous core (Figure 3c,d). Ribs containing adipose tissue in the medullar cavity were also observed, with central blood vessels in the medullar cavity (Figure 3e,f). At the most distal part of the ribs, chondrocytes in the central medulla appeared to be in a proliferative state (Figure 4a). Several samples showed characteristic changes in the medullar cavity, here described as a *transitional zone*, with resorption of the hyperthrophic chondrocytes and appearance of other cell types, that is, erythrocytes, adipocytes and cellular debris (Figure 4b). In addition, samples could show an intact chondocytic medulla with resorption cavities in the lamellar bone (Figure 4c). Anatomically, as in mammals, the ribs were located in close relation to the peritoneum (Figure 4d) with adjacent vessels and nerves (Figure 4e,f).

3.4.2 | Costal changes – Axis deviations and radiolucent medulla

Due to their occurrence in all groups, ribs with axis deviations and radiolucent medulla on radiography were selected from group A for histological investigations. Mild bending of axis showed no pathological changes in the costal anatomy or surrounding soft tissue

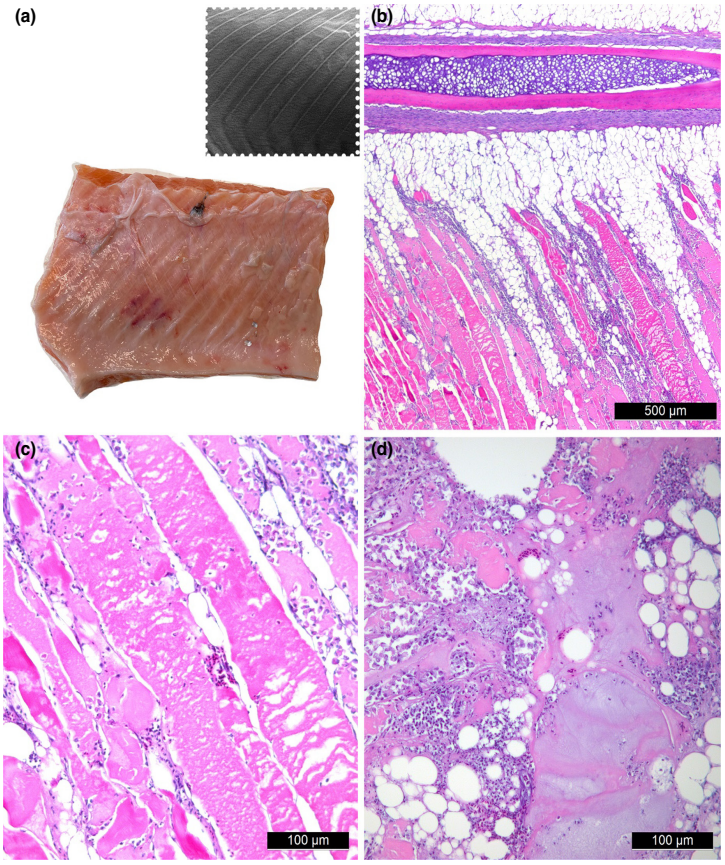

FIGURE 6 Macroscopic, radiographic and histological results of a sample in group B (RFC). (a) Macroscopic evaluation of RFC grade 3 measuring <3 cm, non-pervasive. Stippled square: Radiographic result of area with RFC showing no observable changes in ribs. (b) Histological investigation of area with RFC. No pathological changes in the rib. Neighbouring soft tissue showing degeneration of myocytes and inflammatory cells and haemorrhage. Scale bar: 500 µm. (c, d) Higher magnification (scale bar: 100 µm) of soft tissue inflammation with haemorrhage, degeneration, necrosis and vacuoles present.

(Figure 5a–c). Distal wave shaped ribs were difficult to evaluate in sections, as the rib axis deviated in both lateral/medial and cranial/caudal direction. Nevertheless, the microscopic anatomy of wave shaped ribs was identical to that described in Figure 4a, with proliferative chondrocytes in the medullar cavity (Figure 5d–f). Ribs with radiolucent medulla (Figure 5g,i) on radiography had either a chondrocyttic or an adipocytic medullar cavity, with occational resorption cavities as seen in Figure 4c. No pathological changes were observed in the neighbouring soft tissue.

3.4.3 | Ribs in relation to red focal changes

The histological characteristics of RFC were in line with previous reports (Bjørgen et al., 2015, 2019, 2020), with mild to severe haemorrhage in the soft tissue, necrosis and degeneration of myocytes and infiltration of inflammatory cells. Such findings were not consistently found associated with costal changes (Figure 6a). Herein, affection solely of the adjacent soft tissue at the level of the rib was observed (Figure 6b–d). Other samples contained costal fractures located in the area of the RFC (Figure 7a) but with no affection of the neighbouring soft tissue following histological examination (Figure 7b). Grade 3 RFC could be found without severe costal changes (Figure 7c,d).

3.4.4 | Ribs in relation to melanized focal changes

Group C contained samples of MFC graded from 1 to 3 macroscopically. In radiological investigations, 26 of 55 samples had costal fractures. Sections retrieved from areas containing MFC revealed several different changes histologically, ranging from no histological changes in the bone tissue or neighbouring soft tissue to severe structural changes in both. Histological changes in the soft tissue corresponded to the findings and classification of MFC done by Bjørgen et al. (2019), with changes ranging from mild inflammation and sparse presence of melano-macrophages in the soft tissue to complete loss of skeletal muscle architecture and abundant presence of melano-macrophages, granulomas, fibrosis and inflammation.

As in group B, MFC could have no costal changes on radiology or histology (Figure 8a,b); however, in the adipose tissue of the myosepta near the ribs, melano-macrophages were often found between adipocytes and vessels (Figure 8c,d). Analysis of ribs in MFC could also have similar resorption patterns as seen in samples from group A (Figure 9a–d), but with no apparent affection of the adjacent soft tissue.

When examined by histology, radiographically detected fractures occasionally displayed callus-like structures (Figure 10a–c). Distinct fractures were also observed (Figure 10d–f). These

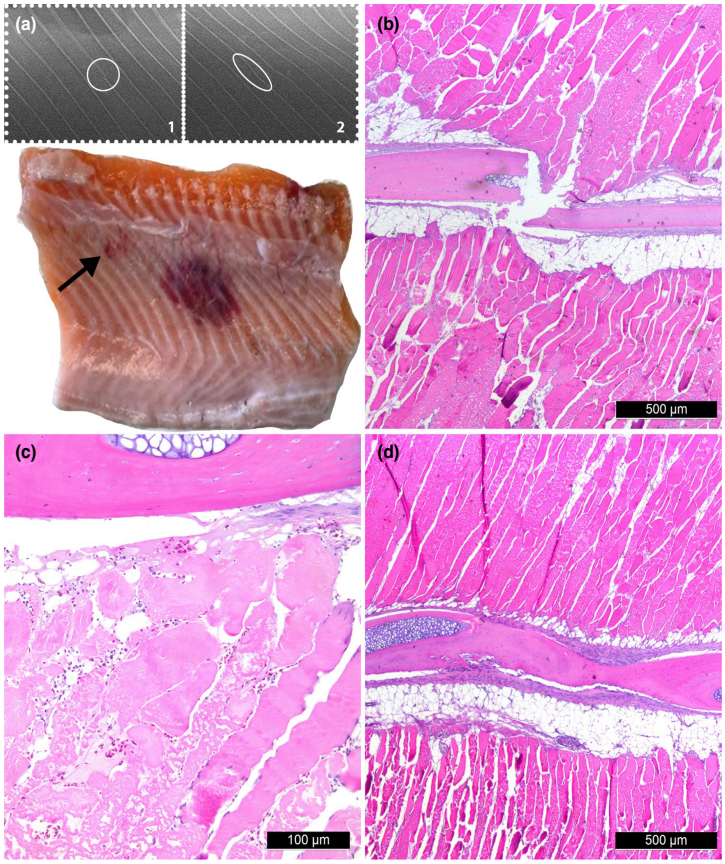

FIGURE 7 Macroscopic, radiographic and histological results of a sample in group B (RFC). (a) Macroscopic evaluation of RFC grade 2 measuring >1 cm (arrow) and RFC grade 3 measuring >3 cm, non-pervasive. Stippled square 1: Radiographic result of RFC grade 2 showing costal fracture (circle). Stippled square 2: Radiographic result of RFC grade 3 showing rib with axis deviation (oval). (b) Histological findings in rib located in area of RFC grade 2. Costal fracture, with no apparent affection of the surrounding soft tissue. Scale bar: 500 μm. (c) Histological findings in RFC grade 3, showing area of soft tissue adjacent to a radiological normal rib with haemorrhage between myocytes and infiltrating inflammatory cells. Degenerated myocytes. Scale bar: 100 μm. (d) Histological findings in rib with axis deviation located in area of RFC grade 3, with no pathological changes in bone structure or in the chondrocyttic core. Adjacent soft tissue is intact. Scale bar: 500 μm.

changes did not consistently correlate with the severity of the MFC (Figure 10d–f); however, samples with grade 3 MFC often showed severe changes in both ribs and adjacent soft tissue (Figure 10g–i). Callus formations were histologically seen as focal thickening of unorganised bone with resorption cavities (Figure 11a–d). Presence of melano-macrophages in adjacent soft tissue was often evident.

4 | DISCUSSION

The rationale for this study was to describe anatomical characteristics of ribs in the Atlantic salmon, and to apply this information in investigating different pathological changes affecting the ribs, and finally, to identify the possible association between costal changes and RFC/MFC. Thus, novel anatomical information provided a basis for the interpretation of different costal changes. Samples of the lateral musculature including ribs were obtained from different groups of Atlantic salmon (wild, farmed and hybrids). The material was categorised by macroscopic evaluation into three different groups: Group A (no macroscopic discolouration), group B (red focal changes/RFC) and group C (melanized focal changes/MFC). All groups were investigated by macroscopical, radiographical and histological analysis.

The samples in all groups were radiographed to reveal possible associations between macroscopic discolouration and costal changes. The variations in rib morphology detected by radiographic investigations coincided with the results of Jiménez-Guerrero et al. 2022 and other radiographic investigations on teleost fish (Fjelldal et al., 2020). As both longitudinal radiolucent ribs and axis variations, that is, mild bending and distal wave shape, were observed in all groups, we assume these costal variations to be of non-pathological nature. Thus, such costal variations detected in fish with macroscopic discolouration and/or inflammation in adjacent soft tissue could be a coincidental finding. Costal fractures and callus formations were commonly found in relation to MFC, indicating that such changes may cause MFC. Alternatively, the chronic inflammatory environment in the neighbouring soft tissue may cause the costal bone tissue to deteriorate, leading to altered rib morphology.

In our study, 40 samples were classified with costal fractures by radiographic examination. Costal fractures were only seen in fish kept in captivity, that is, in on-land tanks and sea water cages. Our results suggest that the cause of costal fractures may lie in the rearing conditions (handling, nutrition etc.), or in the genetic differences between farmed and wild fish (Gjedrem et al., 1991; Gjøen & Bentsen, 1997). However, our wild fish material was limited, and six of ten wild fish samples were not radiographed. Other reports

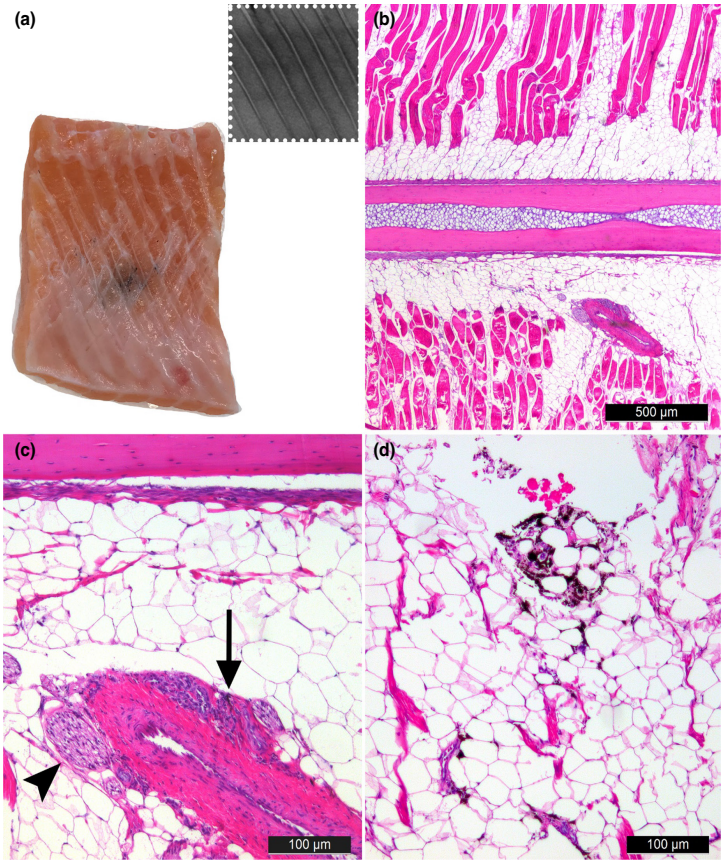

FIGURE 8 Macroscopic, radiographic and histological results of a sample in group C (MFC). (a) Macroscopic evaluation of MFC grade 2, measuring 2 cm, >1 cm deep, non-pervasive. Stippled square: Radiographic results with no observable costal changes. (b) Histological results of area containing MFC. No pathological findings in rib or adjacent soft tissue. A vessel and a nerve are located in close relation to the rib. Scale bar: 500 µm. (c) Vessel and nerve (arrowhead). Melano-macrophages (arrow) in relation to vessel. Scale bar: 100 µm. (d) Melano-macorphages surrounding vacuoles and a vessel in the myoseptal adipose tissue in the area above the rib, towards the peritoneal cavity. Scale bar: 100 µm.

on the prevalence of bone fractures in reared versus wild fish show conflicting results with costal fractures occurring in wild fish (Fjelldal et al., 2020; Jiménez-Guerrero et al., 2022). In commercial aquaculture, skeletal deformities are common and may affect the fish's ability to swim, eat and consequently grow and survive. It is thus of interest to uncover the prevalence and cause of costal fractures, both for animal welfare and production-related interests. As an initial cause of costal fractures in general, trauma is a common and plausible explanation. Incautious rearing conditions may imply rough handling causing local trauma. However, the salmon ribs are well positioned in the myosepta, and the breaking force is expected to be high. Investigations on the mechanical properties of farmed Atlantic salmon ribs showed an increased breaking force following an increase in body weight, pointing out the proximal part of the anterior ribs as the most resistant to breakage (Yao & Mørkøre, 2017). By comparison, farmed rainbow trout showed rib thickness (mm) smaller than that of salmon, but the breaking force is reported to be higher. In this context, it should be noted that the prevalence of MFC in farmed rainbow trout is much lower than in farmed salmon, although most farming conditions are similar (Bjørgen et al., 2015; Mørkøre et al., 2015; Olsen et al., 2021). One other possible scenario is that the force that causes the costal fracture and muscle damage originates from within the abdominal cavity. Following this notion, the ribs of rainbow trout could be strong and stiff enough to protect the underlying musculature, while the weaker and softer ribs of Atlantic salmon could bend and break or bend without fracture, both resulting in a local muscle edema caused by restricted blood flow or bleeding caused by fracture. Such forces could be caused by the swelling of the formulated dry pellets fed to farmed Atlantic salmon, which drinks seawater as part of their natural hypo-osmoregulation (Usher et al., 1988).

As ribs are in close proximity to RFC/MFC, costal changes could be an initial cause of RFC and ultimately MFC. This has recently been addressed by Jiménez-Guerrero et al. (2022). By investigating fillets both with and without macroscopic discolouration, we sought to differentiate costal changes related to discolouration, and changes found in all groups, suggesting the latter being normal variations. Association was not detected between macroscopic discolouration and radiolucent medulla and axis deviations. A statistically significant association was found between RFC/MFC and costal fractures detected by radiographic investigations. However, as histological investigations revealed unaffected ribs in the area of the RFC, and vice versa, these findings underline the importance of histological investigations. MFC were also occasionally found without costal changes, thus the underlying cause of macroscopic discolouration cannot be explained by costal fractures alone.

As with costal fractures, focal bone thickening noted as callus formations were only found in salmon held in captivity. In earlier

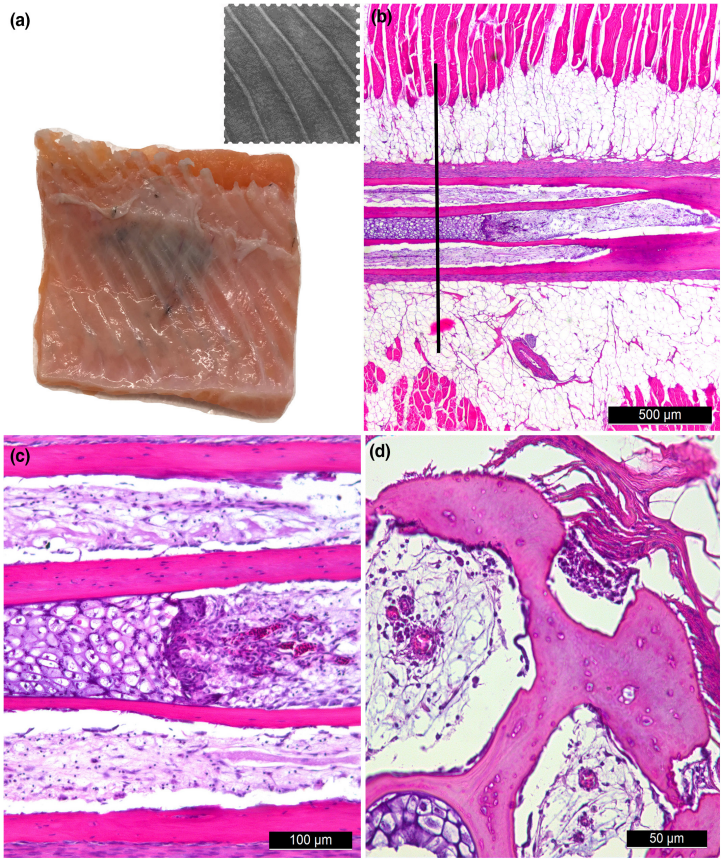

FIGURE 9 Macroscopic, radiographical and histological investigation of a sample in group C (MFC). (a) MFC in sample measuring >3 cm, 2 cm deep, pervasive. Stippled square: Radiographic results of ribs in area of the MFC showing radiolucent medulla in mid part of ribs. (b) Histological characteristics of rib with radiolucent appearance. No pathological changes in surrounding soft tissue. Central medulla of rib showing a transitional zone with removal of hyperthropic chondrocytes. Cavities in the cortical bone adjacent to central medulla showing similar components as the transferred central cavity. Black line: Marked area of cross section in 'd'. Scale bar: 500 μm. (c) Central chondrocyttic medulla with transitional zone. Erythrocytes present post transition. Scale bar: 100 μm. (d) Cross section of area marked with a black line in 'b' showing vessels containing erythrocytes in cavities adjacent to an intact chondrocyttic medulla. Scale bar: 50 μm.

reports, findings of focal bone thickening in teleost bones have been noted as hyperostosis, or swollen bone. Based on several observations, such as species-specific patterns and onset timing of development, hyperostosis in teleosts is regarded a non-pathological expansion of bone (Smith-Vaniz et al., 1995). However, a possible link between hyperostosis and bone fractures has been suggested (Fjelldal et al., 2018, 2020; Jawad, 2013). Fjelldal et al. (2020) discovered a high prevalence of site-specific hemal and neural spine callus-like formations in the axial skeleton in wild ballan wrasse (*Labrus bergylta*), similar to the callus formations in our samples, herein suggesting that a continuous mechanical load may cause the bone to fracture followed by a fracture healing process. Due to the sampling method in our study, we were not able to pinpoint the specific rib (number) in question, as the samples were cropped to include a limited area of the axial skeleton. Thus, site-specific findings were not included in our work. Also, as the size of the hyperostotic structures varies among species and individuals, these structures could in some cases be challenging to distinguish from callus formation only using radiographic investigations. However, due to the association between callus formations and MFC found in our work, it is likely that these changes are the result of a pathological condition in the bone tissue.

By histological investigations, we were able to show anatomical characteristics of Atlantic salmon ribs coinciding with findings in earlier reports, such as osteocyttic bone, longitudinal bone lamella and a chondrocyttic or adipocytic medulla. A transition zone was noted in several ribs. In both wild and farmed fish, multiple longitudinal vessels were found in the adipocyttic medulla post cartilage resorption, and within resorption cavities in the cortical bone with the chondrocyttic core still intact. To our knowledge, such vascularisation of teleost ribs has not been previously described. Resorption cavities in the cortical bone were found both in ribs presented as normal on radiographic investigations, but also in ribs presented with axis deviations and radiolucent medulla. The latter corresponds well with micro-CT results done on thickened salmon rib, herein revealing a thinner compacta with resorptive appearance (Jiménez-Guerrero et al., 2022). As in mammals, bone resorption and vascularisation of bony tissue is a vital part of the bone development and remodelling in teleosts (Benzinou et al., 2002), and resorption activity of both mono- and multinucleated osteoclasts in teleost fish has been well reviewed by Witten and Huysseune (Witten & Huysseune, 2009). In carp and tilapia, Cohen et al. (2012) described an area of woven-like bone structure in the central part of the rib with occasional perforation by the central lumina (Cohen et al., 2012). Interestingly, fish with acellular bone has shown an increase of the hollow medullar cavity correlated with an increase of stiffness of the rib (Horton & Summers, 2009), as is the case in mammals and birds. This might indicate that the resorption of bone in our results is in fact making the

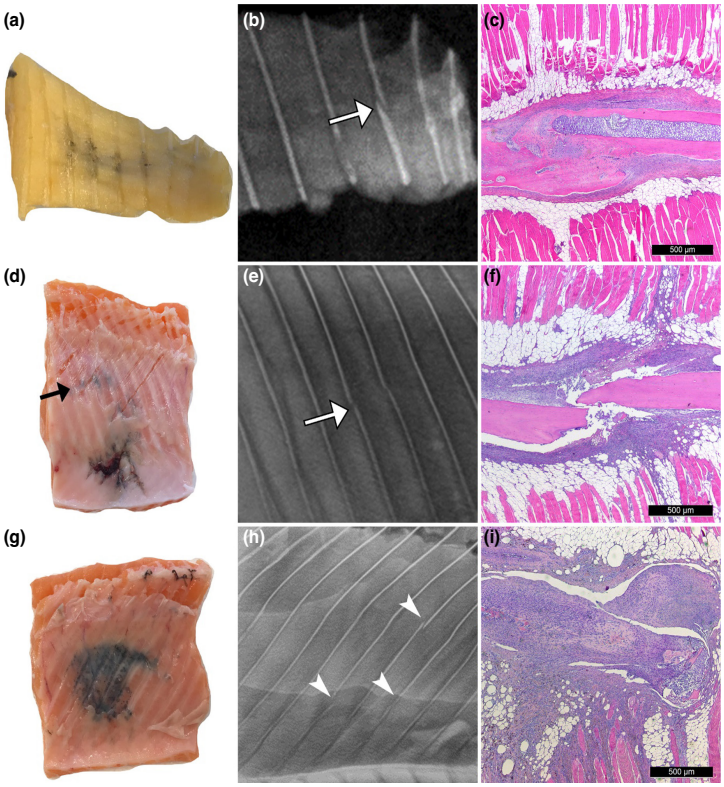

FIGURE 10 Macroscopic, radiographic and histological results of samples in group C (MFC). (a) Macroscopic evaluation of MFC grade 2, measuring >2 cm. (b) Radiographic result of sample in 'a', showing costal fracture (arrow). (c) Histological characteristics of costal fracture seen in 'b'. Loss of normal rib structure, callus-like formation surrounding rib bone collar with an irregular appearance of the longitudinal section of rib. No resorption cavities. No affection of neighbouring soft tissue. Scale bar: 500 µm. (d) Macroscopic evaluation of MFC grade 3, measuring >3 cm, non-pervasive and MFC grade 2, measuring <2 cm (arrow). (e) Radiographic results of sample in 'd', showing costal fracture (arrow). (f) Histological characteristics of costal fracture seen in 'e'. Costal fracture with inflammatory cells present in soft tissue. Adjacent myocytes are degenerated, and melano-macrophages and vacuoles are present in the area of inflammation. Scale bar: 500 µm. (g) Macroscopic evaluation MFC grade 3, measuring >3 cm, 2 cm deep, non-pervasive. (h) Radiographic results of sample in 'g', showing costal fractures (arrowheads). (i) Histological investigations of area containing costal fractures in 'h'. Total loss of normal tissue arcitecture in rib and adjacent soft tissue. Inflammatory cells are present, in addition to melano-macrophages and vacuoles. Degeneration of myocytes is evident. Scale bar: 500 µm.

rib more resistant by adapting to changing stressors, a trait beneficial to the fish. If so, this corresponds to the increased breaking force following increased body weight (Yao & Mørkøre, 2017). However, it is noteworthy that cross sections of the ribs revealed an irregular appearance of the outer circumference in addition to large resorption cavities in the bone, resulting in areas of distinctly thinner cortical bone. There is limited knowledge on osteoporosis in farmed Atlantic salmon; however, osteoporotic conditions can be induced by several factors in teleost species such as zebrafish (Rosa et al., 2021), a fish widely utilized in research revolving human medicine. We speculate if the resorptive changes in Atlantic salmon rib are pathologically induced, thus affecting the rib breaking force negatively, or if this is merely a physiological and anatomical trait in salmon due to life in an aquatic milieu.

Co-occurring resorption and bone formation is vital for healthy bone growth, thus as the fish grows, bones will subsequently increase in size. Ribs with radiographical radiolucent medulla were detected in all groups, but not in all samples. As these findings were often located in proximal and mid parts of the ribs, we speculate that the increased thickness of the rib due to growth is what allows the X-ray beam to pass through tissue that are of less radiopaque properties, giving the medulla a radiolucent appearance. The size of the individual fish was not registered in our work; however, we believe that the fish size will impact the findings of radiolucent medulla.

Histological investigations of the most distal part of ribs, both normal and wave shaped ribs, revealed a central medulla containing proliferative chondrocytes. These results are in line with Soliman (2018), showing chondrocytes arranged as resting, proliferating and hypertrophic zones in the medullar cavity in both the proximal and distal part of the rib. In our work, we were not able to reveal a proximal growth plate in the ribs. Wave shaped ribs were solely located at the distal end of the rib, surrounded by abundant

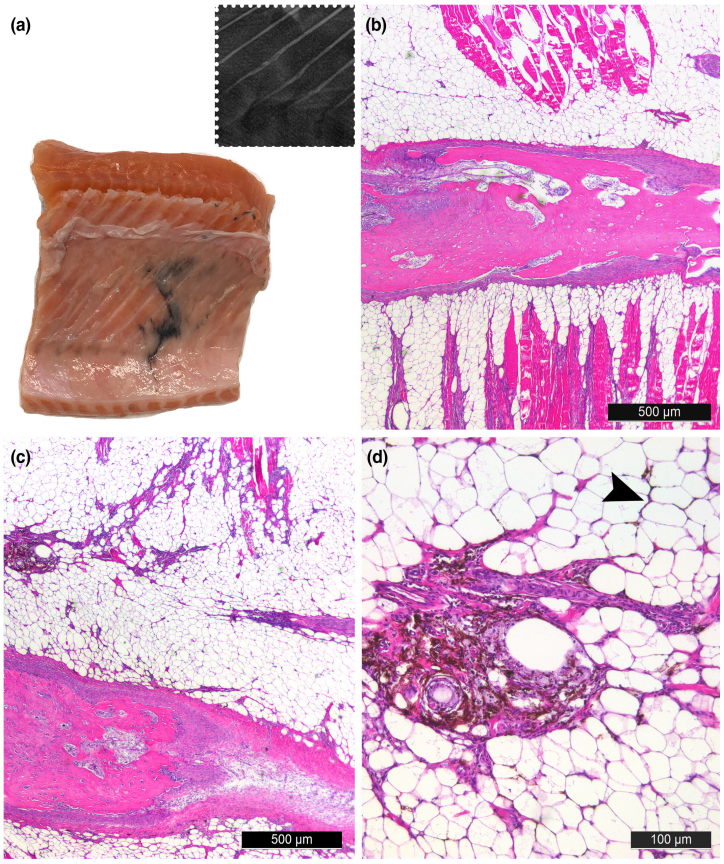

FIGURE 11 Macropscopic, radiographical and histological investigation of a sample in group C (MFC) presented with focal thickening of ribs detected on radiographic investigations. (a) MFC measuring >3 cm in length, non-pervasive. Stippled square: Radiographic results showing thickened, irrgular shape in distal regions of ribs. (b) Histological findings in thickened and irregular rib located in area of melanized focal change. Normal bone tissue arcitecture is lacking, with focal bone resorption. Adjacent soft tissue with infiltrating inflammatory cells and vacuoles. Scale bar: 500 µm. (c) Adjacent soft tissue dominated by adipocytes, with melano-macrophages present. Scale bar: 500 µm. (d) Granuloma with abundant presence of surrounding melano-macrophages. Melano-macrophages are also present between adipocytes (arrowhead). Scale bar: 100 µm.

adipose tissue and to a lesser extent skeletal muscle tissue. We speculate that this axis deviation is due to the anatomical structure and position, as muscle attachment surely will anchor the ribs and restrict their shape in more proximal areas; however, ribs noted as wiggled have also been observed in Atlantic salmon with phosphorus deficiency (Baeverfjord et al., 1998). No pathological changes were found in the cortical bone or surrounding soft tissue of wave shaped ribs in group A; however, in samples with MFC located at the equivalent areas, neighbouring soft tissue could be severely affected. In terms of other axis deviations, histological investigations on mild bending of axis in group A revealed normal bone morphology and surrounding soft tissue, with a mild curve in the rib axis. Thus, supported by radiology and statistics, both axis deviations found in our work are suggested to be normal variations of rib morphology in at least some teleost species, including Atlantic salmon. However, these results cannot rule out that inflammation in the soft tissue can alter the axis of the rib, or that these deviations show different breaking force than other costal changes, as resorption of the cortical bone followed by vascularization was indeed seen in all parts of the rib, including the distal wave shaped part.

As Moss pointed out in 1961 (Moss, 1961), the vascular pattern in teleost bone varies substantially among species and even within one individual. Our findings show several longitudinal vessels within the ribs, thus the ribs in Atlantic salmon have great potential for haemorrhage and the recruiting of haematopoietic and inflammatory cells in the event of a fracture. The histological investigations conducted in our study showed that costal fractures could be accompanied by acute inflammation and haemorrhage in the adjacent muscle and connective tissue. This complies with mammalian fracture repair explained by a four-stage model, where a fracture will cause haemorrhage in the neighbouring soft tissue, followed by acute inflammation, callus formation and remodelling of the callus (Kolar et al., 2011; Marsell & Einhorn, 2011; Schindeler et al., 2008). In Moss' work on fracture repair in acellular and cellular teleosts (Moss, 1962), the examined opercular and lower jaw elements were sparsely vascularised, thus a hematoma was not a dominating feature in the fracture healing process. In our work, costal fractures were occasionally found with no changes in the neighbouring soft tissue, indicating that the fracture occurred postmortem. Importantly, some samples with focal macroscopic discolouration contained costal fractures in the equivalent area presented by radiography; however, histological investigations revealed postmortem fractures, thus the macroscopic focal discolouration herein must have a different origin.

In mammalian fracture healing, bone splints are covered by a soft and later a hard callus. We show that ribs with callus-like appearance in radiology were histologically presented with corresponding callus

properties, that is, woven bone with resorption cavities covering areas of what was presumed to be an old fracture site. This is in line with earlier reports on fracture healing in teleosts using both radiography and histology (Fjelldal et al., 2018; Geurtzen et al., 2014; Moss, 1962; Takeyama et al., 2014).

In sum, our study has shown variations in the anatomical characteristics in Atlantic salmon ribs, including vascularisation and resorption cavities. This included ribs with intact chondrocyttic medulla and no vessels or resorption cavities present, and ribs with multiple longitudinal vessels in both the medullar cavity and in resorption cavities in the cortical bone. Our results provide additional knowledge to the association between costal changes and RFC/MFC found in earlier reports and has demonstrated the potential for local haemorrhage in the event of costal fractures. In accordance with earlier findings, the initial cause of RFC/MFC cannot be fully explained by costal changes alone.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study (histological slides and X-ray files) are available from the corresponding author upon reasonable request.

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SHORT COMMUNICATION

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Diffuse melanization of the red skeletal musculature in farmed Atlantic salmon (*Salmo salar* L.)

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1 | INTRODUCTION

Farmed Atlantic salmon (*Salmo salar* L.) are susceptible to melanization of the white muscle ('the filet'), where melanized spots have a prevalence of 20%–30% at slaughter (Mørkøre et al., 2015). More than 90% of melanized changes in the filet manifest as focal discoloration in the cranio-ventral part (Bjørgen et al., 2019). These changes develop from focal haemorrhages, that is, red focal changes, which are the initial and acute stage of the condition (Bjørgen et al., 2015, 2019). The haemorrhages are believed to progress into a chronic inflammatory condition with melanization (Koppang et al., 2005; Larsen et al., 2012). Although the development of severe melanized focal changes has been associated with persistent PRV-1 infection (Bjørgen et al., 2019), the initial causes of red focal changes are unknown (Bjørgen et al., 2020). The focal appearance and restricted localization argue for underlying anatomical predispositions, and hypotheses such as fat accumulation and fat-induced inflammation, local ischaemia and costal abnormalities have all been proposed as hypotheses (Bjørgen et al., 2019; Jiménez-Guerrero et al., 2022). Still, no single cause has been pinpointed and the aetiology of the condition is probably of multifactorial origin.

Melano-macrophages are common immune cells in fish and are normally found in abundance in lymphoid organs such as kidney, spleen and liver (Bjørgen & Koppang, 2021; Thorsen et al., 2006). They are especially active in chronic, granulomatous inflammation and can thus be found in a range of different inflammatory conditions (Koppang et al., 2005; Poppe & Breck, 1997). Although focal melanization is the most prevalent manifestation in white muscle, other forms have been reported, including melanization near the vertebrae (Trangerud et al., 2020), in the dorsal part of the fillet and diffuse melanization of the dorsal and ventral white muscle (Ugrovatov, 2016). In summary, the white muscle could seem more disposed for inflammatory conditions attracting melano-macrophages. On the contrary, melanization is rarely reported to occur in the red skeletal muscle but has occasionally been observed by producers or customers (Personal observation, Line Rønning, Lerøy Seafood Group). As the red skeletal muscle is found on the lateral side of the trunk (reviewed by Kiessling et al., 2006), beneath the skin, melanization is often left undiscovered due to the fileting methods. Although conditions such as wounds and foreign bodies can induce the reaction of melano-macrophages (Roberts et al., 1973), melanization of the red skeletal muscle has not been reported as a quality concern.

Here, we report the appearance of melanization in farmed Atlantic salmon affecting solely the red skeletal muscle. We provide histopathological description of the condition and discuss possible causes of this presumed uncommon condition.

2 | MATERIALS AND METHODS

2.1 | Fish origin and health

In November 2016, approximately 52,000 harvest-ready Atlantic salmon from Lerøy's location Kjørvikgrunn were slaughtered according to production standards. The fish was of the AquaGen QTL IPN/PD breed and had an average weight of 4613 g. All fish had been vaccinated with Alpha Ject micro 6 (Pharmaq, part of Zoetis).

Heart and skeletal muscle inflammation (HSMI) caused by PRV-1 infection was confirmed by histology in November 2015. Salmonid alphavirus 2 (SAV2) was detected in the population by RT-qPCR in July 2016 and Pancreas disease (PD) caused by SAV2 infection was confirmed by RT-qPCR and histological investigations in August 2016. Cardiomyopathy syndrome (CMS) caused by piscine myocarditis virus (PMCV) was suspected in August 2016. This was confirmed by histological investigations in December 2016. Increased mortality was registered in relation to all three disease outbreaks.

2.2 | Sampling and RT-qPCR

At slaughter, a high prevalence of melanization in the red skeletal muscle was detected. Affected (n = 6) and unaffected fillets (n = 6) were collected at the production line and shipped on ice to the School of Veterinary Medicine, Adamstuen, Oslo, Norway. The changes were photographed, and samples were collected from three different areas of the filet (cranial, mid, and caudal parts, see Figure 1) and put in buffered formalin. Both cross-sectional and longitudinal samples were obtained. From each fish, a sample collected from the red muscle (mid part) was put in RNAlater and sent to PatoGen AS, Ålesund, Norway, for RT-qPCR analysis for common viral infections affecting the skeletal muscle: Piscine orthoreovirus 1 (PRV-1) and salmonid alphavirus 2 (SAV2). The samples were also analysed for infectious pancreas necrosis virus (IPNV). The RT-qPCR analyses are accredited and validated to ISO7025 standards. Details of purification method and PCR conditions could not be disclosed by PatoGen due to issues related to competing patents. Samples were defined as positive when having a PRV-1, SAV2 or IPNV C_t lower than 37.0. Statistical analysis of PRV-1 data was performed using Mann–Whitney U-test in STATA (StataCorp. 2019. Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC).

2.3 | Histological investigations

The samples were embedded in paraffin and stained according to standard procedure with haematoxylin and eosin. In addition, all samples were stained for detection of fibrin according to Lendrums Maritus Scarlet Blue (MSB) staining protocol, Giemsa staining to differentiate nucleus morphology and cytoplasm of blood cells, Van Gieson (VG) for detection of collagen and Gram staining for detection of Gram-positive/negative bacteria.

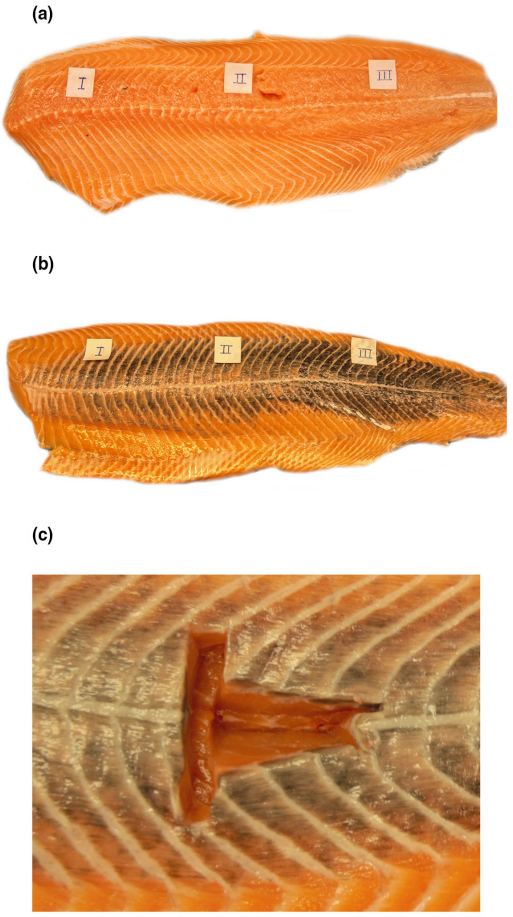

FIGURE 1 Macroscopic appearance of (a) control filet, (b) melanized filet, lateral projection (skin removed) and (c) clear demarcation in melanization of red skeletal muscle presented through longitudinal and cross sectioning. Note the shallow layer of discoloration, reflecting only affection of the red muscle layer and not the underlying white musculature. Samples were collected from cranial (I), mid (II) and caudal (III) parts.

3 | RESULTS

3.1 | Macroscopic evaluation

Severe, diffuse melanization of the red skeletal muscle was observed as superficial, black discoloration in the fillet of the affected fish (Figure 1). The extent of the discoloration was identical to the anatomical placement of the red skeletal muscle. In transverse sections of the fillet, a clear demarcation in melanization confirmed that solely the red, and not the white skeletal muscle

was affected (Figure 1c). All the control individuals were devoid of discoloration.

3.2 | Histological analysis

In H&E-stained sections, the macroscopically observed demarcation of melanization between white and red muscle was confirmed, as the white muscle had normal appearance while the red muscle was severely affected (Figure 2a). The changes in the red muscle were dominated by muscle fibre degeneration and fibrosis. Fibrosis was confirmed by VG stain (Figure 2b). Scattered immune cells, predominantly macrophage-like cells were observed and melano-macrophages and what appeared as either large accumulations of melanin or collections of melano-macrophages were detected (Figure 2c). Some muscle fibres showed signs of regeneration, including cytoplasmic basophilia and rowing of nuclei (Figure 2d). The histological features were similar in all affected fish and for the different areas sampled. Giemsa and Gram staining did not show presence of bacteria.

No pathological changes were detected in the control fish (Figure 3a,b); however, scattered melano-macrophages were occasionally detected in the endomysium of red muscle. (Figure 3c,d).

3.3 | RT-qPCR

Tissue from the mid part of the filet was analysed for the presence of PRV-1 and SAV2 (Table 1) but also for IPNV, by RT-qPCR, as IPN is a differential diagnosis to PD. All affected samples were positive for PRV-1 with a median C_t value of 30.2, for the control fish, four samples were PRV-1 positive, and two were negative. When the negative value is set to 37, that is, cut-off for the RT-qPCR, there were statistically significant difference between the C_t values for PRV-1

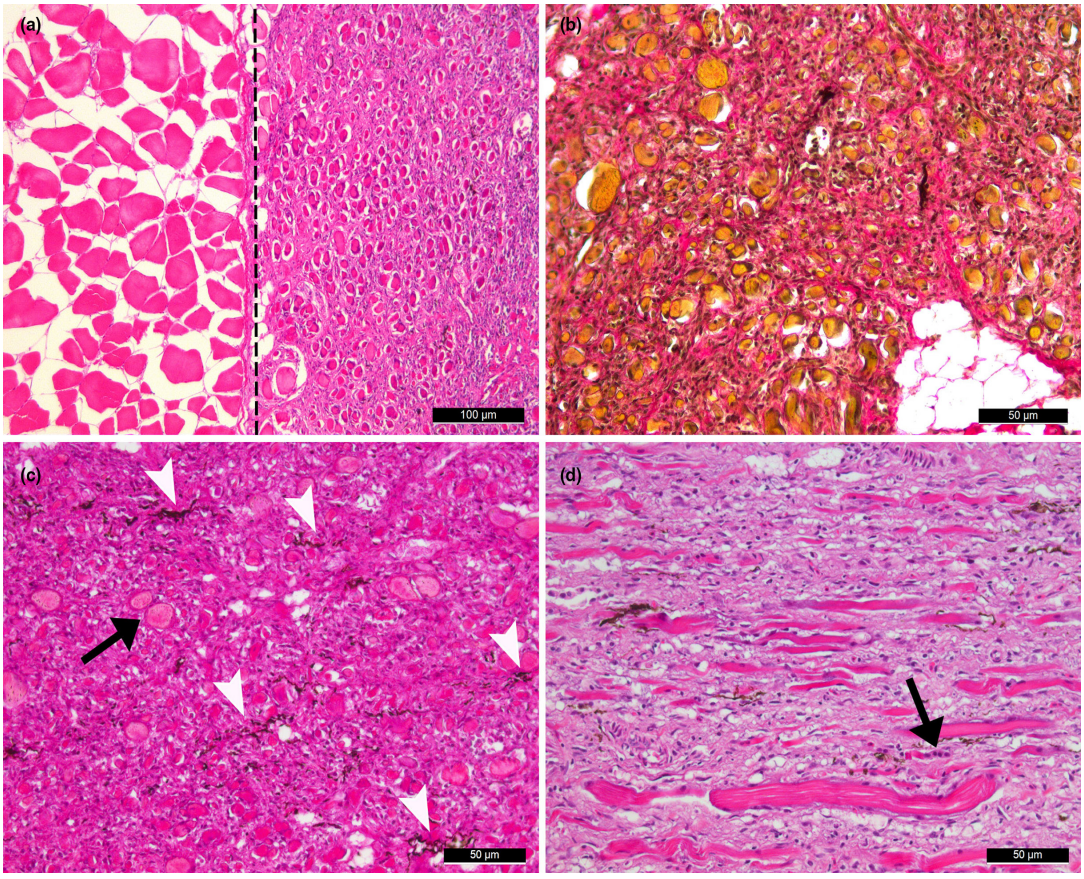

FIGURE 2 Histological analysis of red muscle, affected fish. (a) HE stain. Demarcation between unaffected white muscle (left) and affected red muscle (right). Fixation artefacts, seen as myocyte shrinkage with peripheral empty space, are evident in white muscle. (b) VG stain confirming the presence of collagen (pink). (c) HE stain, cross section. Red skeletal muscle with degenerated muscle cells (black arrow), extensive fibrosis and infiltrates with immune cells. Melano-macrophages or melanin accumulations scattered within the red muscle (white arrowheads). (d) HE stain, longitudinal section. Muscle fibres in different size/stages of regeneration, supported by occasionally basophilic fibres and central rowing of nuclei (black arrow).

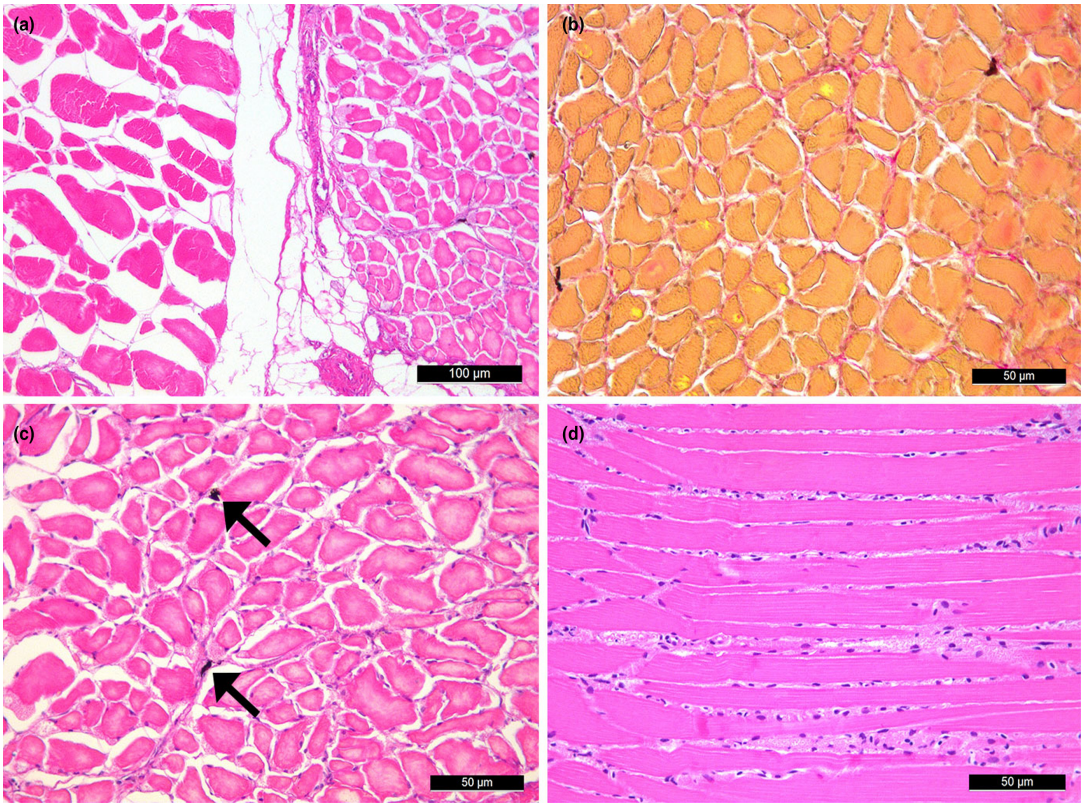

FIGURE 3 Histological analysis of red muscle, control fish. (a) HE stain. White (left) and red (right) skeletal muscle fibres separated by a septum with predominantly fat-containing cells. (b) VG stain showing intact muscle cells (yellow) and collagen (pink) in endomysium. Note the sparse amount of collagen. (c) HE stain, cross section. Muscle cell diameter 25–45 µm. Melano-macrophages (arrows) in the endomysium. (d) HE stain, longitudinal section. Striated myocytes with multiple peripheral nuclei.

for affected versus non-affected groups ($p = .0022$). SAV2 was detected in three affected samples, of which one had a C_t 23.2, and in two non-affected samples. No samples were positive for IPNV.

4 | DISCUSSION

In this case report, we present melanization affecting the whole red skeletal muscle in farmed Atlantic salmon. The condition, that in general is considered as a rather rare quality and possibly welfare problem, was detected during quality control after filleting, as a black longitudinal sheath directly underneath the skin.

Histological analysis revealed diffuse inflammation with infiltrates of inflammatory cells where melano-macrophages were abundant. There was extensive fibrosis. In general, there were several histological similarities with the more common melanized focal changes; however, no granulomas were detected in the red skeletal muscle. Granulomas are hallmark traits for severe melanized focal changes in the white muscle and typical for some chronic inflammatory conditions (Koppang et al., 2005; Shah et al., 2017). The absence of organized granulomas may imply that the underlying cause differs from that of the melanized focal changes in white muscle. Alternatively, anatomical and physiological conditions in red muscle could contribute to the development of the changes seen in our study. Nevertheless, granulomatous inflammation in red muscle has been reported in rainbow trout affected by proliferative kidney disease (PKD) (Fernandez-De-Luco et al., 1997); thus such an immune reaction can also occur in red muscle. Signs of regeneration were seen in most changes in our work, indicating that the condition is temporary and heals over time. Macrophage-like cells and myophagocytosis were observed in the affected red muscle, but scattered melano-macrophages were also detected in the endomysium between intact muscle fibres from non-affected fish. Melanin is rarely observed in the red skeletal muscle in routine diagnostics (personal communication, Dr. Hege Hellberg, Pharmaq Analytic, Norway); however, this could be a normal trait of slaughter-ready farmed salmon, or alternatively the scattered melano-macrophages could be remnants from a preceding regeneration and clearing of the condition.

Red muscle is anatomically different from white muscle in several important aspects. Although the red muscle makes up <10% of the total muscle mass in Atlantic salmon, it is important

TABLE 1 RT-qPCR

	Fish	PRV-1	SAV2
Affected	1	28.8	28.1
	2	29.6	23.2
	3	31.8	31.6
	4	27.6	n.d.
	5	30,8	n.d.
	6	33.0	n.d.
Non-affected (controls)	7	34.8	n.d.
	8	34.6	n.d.
	9	n.d.	29.4
	10	35.5	n.d.
	11	35.2	n.d.
	12	n.d.	33.1

Note: C_t values for PRV-1 and SAV2. Threshold PRV1: 37 and SAV: 37.
Abbreviation: n.d., not detected.

in enduring and slow movement as it is rich in mitochondria and heavily vascularized, and thus adapted towards aerobic exercise (Anttila & Manttari, 2009; Kryvi & Poppe, 2016). White muscle, on the other hand, generates speed and extreme anaerobic movements, but has a relatively poor blood supply and only scattered mitochondria (Hudson, 1973; Nag, 1972). These differences could have an impact on the process of regeneration and healing in red and white muscle which subsequently can lead to different forms of melanization.

Of the viruses commonly affecting the skeletal muscle in salmon, some are prone to cause red muscle damage (Bruno et al., 2013). PRV-1, which is the cause of HSMI may specifically affect the red muscle and induce myositis, and although it is not a common reaction, melano-macrophages have been described in the red skeletal muscle of HSMI diseased fish (Wessel et al., 2017). SAV2 and 3, which are the causes of PD in farmed salmon in Norway, can affect both red and white muscle, typically late in the course of the disease development (Bruno et al., 2013). The endomysial inflammation and fibrosis observed in our material could result from both SAV2 and PRV-1 infection. One could speculate that SAV2 was the key contributor due to the ongoing outbreak concurrent with slaughter; however, SAV2 was only detected in three of the six affected samples. The C_t values for PRV-1 of affected fish were statistically significantly lower ($p = .0022$) than from non-affected fish. Although the RT-qPCRs were run without a normalizing RNA reference, and the amount of RNA thus cannot be quantified, the C_t values of PRV-1 reflects in general that the load of PRV-1 was significantly higher in melanized red muscle than in normal red muscle tissue. This indicates a correlation of the amount of PRV-1 and the condition, but it does not necessarily specify causation.

All affected fish were infected with PRV-1, including the three SAV2 infected. The two SAV2 infected non-affected fish were negative for PRV-1 by RT-qPCR. The number of samples here is very small, but it could be speculated that the findings indicate that SAV2 infection alone does not induce red muscle melanization. The co-infection with PRV-1 and SAV2 found in three of six affected samples is difficult to speculate upon. The fish came from a population where HSMI and PD had been confirmed prior to slaughter, that is, the population had probably gone through serious infections with both PRV-1 and SAV2. In addition, CMS caused by PMCV was suspected and later detected at the location; however, PMCV does not infect skeletal muscle. Melanization is a general inflammatory response where one important function is to protect the surrounding tissue against oxidative damage (Larsen et al., 2012). Melanization has been detected not only in association with intraperitoneal injections (Koppang et al., 2005; Poppe & Breck, 1997) but also in several viral infections, for example, PRV-1 has been associated with severe melanized focal changes in the white skeletal muscle (Bjørgen et al., 2015, 2019) and melanization of the heart atrium has been reported in fish with CMS (Fagerland et al., 2013). In an epidemiological study, PD diagnosis was correlated with increased prevalence of melanized focal changes in the filet (Lund et al., 2018). Although statistical analysis of our material could indicate that PRV-1 infection is associated with red muscle melanization, other infections or even co-infections with SAV2 or PMCV may have contributed to the development of the condition.

In situ detection of viruses in the red muscle could have added information about the presence of PRV-1 and SAV2 in relation to the histopathological changes, but this was not possible to perform due to sub-optimal fixation of the field material. There is no experimental model that are known to induce the condition, and one is dependent on samples collected at the slaughter line in field.

In conclusion, we have reported a case of melanization solely affecting the red skeletal muscle of farmed Atlantic salmon. The condition has unknown aetiology and histologically appears diffuse and not granulomatous as is the dominant form in melanized focal changes in the white muscle. The involvement of SAV2 and PRV-1 infection might participate in the development of the changes and should be a target in future research if the condition develops into a more generalized problem for the industry.

AUTHOR CONTRIBUTIONS

MB: carried out histological analysis and wrote the manuscript. LR: sampled field material and commented on the manuscript. ER: commented on the manuscript. EOK: conducted sample preparations and histological experiments, commented on the manuscript. HB: conducted sample preparations and histological experiments, commented on the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study (histological slides) are available from the corresponding author, HB, upon reasonable request.

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RESEARCH ARTICLE

Red and melanized focal changes in white skeletal muscle in Atlantic salmon (*Salmo salar*): Comparative analysis of farmed, wild and hybrid fish reared under identical conditions

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Abstract

Selective breeding plays a vital role in the production of farmed Atlantic salmon and has shown success in many aspects. Still, challenges related to fish health and welfare continue to result in significant economic losses. One such challenge is red and melanized focal changes (RFC/MFC), which result from acute and chronic inflammation, respectively, in the skeletal muscle. Importantly, RFC/MFC has not been observed in wild Atlantic salmon, suggesting that both external and genetic factors may contribute to the development of inflammation. To investigate the underlying cause of RFC/MFC, we conducted a study involving 1854 Atlantic salmon of farmed, wild and hybrid origin. All fish were reared under identical conditions to minimize the influence of external factors. Throughout the production cycle, the fish was monitored for growth parameters and examined for RFC/MFC using macroscopic and histological analysis. We found no association between the experimental groups and the presence of RFC/MFC. Histological investigations revealed melano-macrophages in the soft tissue in freshwater smolt, although no macroscopic discoloration was observed. MFC showed granulomas in various stages, suggesting a complex progression of the condition. In summary, we conclude that RFC/MFC is primarily caused by external factors found in the rearing facilities of farmed Atlantic salmon.

KEYWORDS
black spot breeding, melanin, melano-macrophage, quality

1 | INTRODUCTION

The farming of Atlantic salmon (*Salmo salar*) has undergone significant advancements and intensification in production. In 2022, approximately 450,000 tonnes of salmon were exported from Norway, with a value of 40 billion NOK (Norwegian Seafood Council, 2023). Over the past five decades, breeding programmes have been developed and implemented to enhance the suitability of the species for production (Gjedrem, 2010; Gjedrem, Gjøen, & Gjerde, 1991). These breeding efforts, spanning several generations, have focused on selecting traits such as improved growth, delayed sexual maturation, optimal fat content, high-quality fillets and enhanced immunity against infectious agents (Gjedrem, 1983; Gjedrem, Salte, & Gjøen, 1991; Gjøen & Bentsen, 1997; Houston et al., 2008).

Consequently, today's farmed strains, cultivated by companies like Mowi AS, Salmobreed AS and Aquagen AS, exhibit rapid growth and increased resistance against disease. Initially, breeding relied on phenotypic mass selection, progressing to family-based selection, marker-assisted selection and currently genomic selection. The targeted breeding for domesticated individuals has led to genetically distinct farmed strains that differ from their wild counterparts (reviewed by Glover et al., 2017).

Despite extensive research efforts dedicated to understanding the biology and genetics of Atlantic salmon, and to develop breeding programmes, commercial production still faces significant challenges related to health and quality. One major concern in production is the occurrence of melanized focal changes (MFC), predominantly found in the cranioventral region of the white skeletal muscle (Bjørgen et al., 2019). On average, these changes are present in 20% of all fillets produced in Norway (Mørkøre et al., 2015). Additionally, melanization has been reported, albeit less commonly, in the dorsal parts of the fillet and in the red skeletal muscle (Brimsholm et al., 2022). It is believed that MFC originate from red focal changes (RFC), which are acute changes characterized by haemorrhages and muscle necrosis (Bjørgen et al., 2019, 2020). These muscle bleedings can progress into chronic, non-resolving granulomatous inflammation with an abundance of melano-macrophages (Larsen et al., 2012). The most severe changes have been associated with the presence and replication of piscine orthoreovirus 1 (PRV-1) (Bjørgen et al., 2015, 2019). Although PRV-1 is not considered to be the initial cause of the RFC, it is suggested that the virus, which infects Atlantic salmon erythrocytes (Finstad et al., 2014), is internalized by macrophages during the acute inflammatory response, where it subsequently can avoid elimination, leaving the phagocytes persistently infected (Malik et al., 2019). This, in turn, may lead to a chronic, granulomatous inflammation with organized granulomas, as the immune system attempts to 'wall off' the infection (Bjørgen et al., 2015). Various theories have been proposed to explain the cause of RFC/MFC, including external trauma, vaccination and costal fractures (Brimsholm et al., 2023; Jiménez-Guerrero et al., 2022; Koppang et al., 2005). Impaired oxygen transport resulting from abnormal heart morphology and function in farmed salmon (Johansen et al., 2017; Poppe et al., 2003) has also been suggested as a potential cause for ischemia and necrosis of myocytes. In summary, the initial cause of RFC/MFC appears to be multifactorial, and no single cause has been conclusively identified so far.

The absence of RFC/MFC in wild salmon suggests the involvement of both genetic and environmental factors in the development of this condition. Various studies comparing farmed, wild and hybrid salmon have highlighted the significant impact of genetic factors on morphological and physiological differences, such as growth rate, condition factor and plasma growth hormone (GH) level (Debes et al., 2021; Fleming et al., 2002; Glover et al., 2009; Thodesen et al., 1999). Importantly, genetic variations have been shown to influence susceptibility to infections in Atlantic salmon (Glover et al., 2004, 2017; Lawlor et al., 2009; Lush et al., 2019; Moen et al., 2009). However, to date, no peer-review reports have explored the hereditary variation within Atlantic salmon genotypes and its relationship with the prevalence of MFC.

In this study, we employed a common-garden experimental design to assess the contribution of hereditary factors to RFC/MFC development in Atlantic salmon. Our investigation included individuals from farmed, wild and hybrid populations, spanning from the parr stage to the final slaughter size. We observed and documented the presence of RFC and MFC and conducted histological analyses to examine the variations in these changes across different fish groups. Furthermore, we collected data on growth parameters and heart size in all fish groups to explore potential relationships and interactions associated with the prevalence and development of RFC/MFC.

2 | MATERIALS AND METHODS

2.1 | Fish origin and experimental groups

In this study, a total of 1854 Atlantic salmon were included. Genetically wild (originating from naturally bred), farmed (originated from parents bred by selection) and hybrid salmon (crossed between wild and farmed) were kept together in the same cages under standardized rearing conditions at the Matre and Solheim facilities, Institute of Marine Research (IMR), Matredal, Norway, ensuring identical environmental factors. Research done on farmed, wild and hybrid salmon using the same common-garden approach has been conducted at these facilities for more than a decade (Debes et al., 2021; Perry et al., 2019; Solberg et al., 2013). Wild fish were characterized by first-generation offspring from wild salmon parents' eggs and sperm (Etne × Etne) caught in the river Etneelven, Etne, Norway. Hybrids represented combinations of wild and farmed salmon, with both combinations included as follows: wild salmon eggs crossed with farmed salmon sperm from the Mowi strain (Etne × Mowi = Hybrid1) and farmed salmon eggs from the Mowi strain crossed with wild salmon sperm (Mowi × Etne = Hybrid2). Farmed salmon were represented by farmed salmon eggs and sperm of the Mowi strain (Mowi × Mowi). The four experimental groups were kept in separate hatching containers, with artificial hatching substrate (astroturf) at a maximum temperature of 8°C (Hansen & Møller, 1985). The experimental groups were further kept separate in 90 × 90 × 50 cm freshwater tanks until they reached approximately 5 cm in length. The fish was then transferred to four 1.5 × 1.5 × 0.7 m freshwater tanks, holding mixed experimental groups. At the trial start-up in March 2018 (day 0 of trial), with a fish length of 13–15 cm (±35 g), a 12 mm PIT tag was injected into the abdominal cavity combined with measurements of growth parameters (Table 1). The fish were vaccinated with intraperitoneal injection in March 2018 using AquaVac 6 Vet (Manufacturer: MSD Animal Health. Inactivated agents: IPNV, serotype Sp, *Aerormonas salmonicida ssp. Salmonocida*, *Vibrio salmonicida*, *Listonella (Vibrio) anguillarum* serotype O1, *Listonella (Vibrio) Anguillarum* serotype O2a, *Mortiella viscosa* (MSD Animal Health, 2023). In May 2018 (day 67 of trial), the yearling smolts were transferred to 5 m diameter seawater tanks after sampling (Table 1). Fish from all experimental groups were transferred

TABLE 1 Sampling groups and time points.

Month/year	Day of trial	Fish status	Origin	Growth parameters	*n*/m/h
March 2018	0	Parr	FW tanks (sep)	Measured	1854/0/0
May 2018	67	Smolt	FW tanks(sep)	Measured	1787/20/20
May 2018	SW tank transfer	Smolt	SW tanks(mix)	ns	ns
August 2018	SW cage transfer	Post-smolt	SW cage (mix)	ns	ns
March 2019	369	Salmon	SW cage (mix)	Measured	804/804/46
September 2019	537	Salmon	SW cage (mix)	Measured	669/627/68

Abbreviations: FW, freshwater; h, number of fillets from (m) sampled for histological evaluation; m, number of fish sampled for macroscopic evaluation of fillet; mix, experimental groups are mixed; *n*, number of fish measured for growth parameters; ns, no sampling; sep, experimental groups are kept separate; SW, sea water.

to two 12 × 12 × 14 m seawater cages at the IMR Solheim aquaculture facilities in August 2018 and kept jointly in the cages. The maximum biomass was observed at the end of the trial (day 537), with a stocking density in each seawater cage at 0.7 kg/m^3. The fish was reared until the third sampling in March 2019 (day 369 of trial) and the fourth sampling in September 2019 (day 537 of trial). Removal of salmon lice (*Lepeophtheirus salmonis*) was performed manually. At sampling 1 and 2, the fish was anaesthetized with Finquel Vet 0.1 g/L (Manufacturer: MSD Animal Health) prior to measurements. At sampling 2, 3 and 4 the sampled fish were killed with an overdose of Finquel 0.5 g/L and bled out.

2.2 | Ethical considerations

All handling procedures of fish complied with the EU (2010/63/EU) and Norwegian legislation. This animal study was reviewed and approved by Animal Care and Use Committee/IACUC and NARA (permit number 15780) according to the European Union Directive 2010/63/EU and Norwegian regulation FOR-2015-06-18-761.

2.3 | Sample handling and macroscopic evaluation

Production parameters, that is body weight (grams), length (centimetres from snout to the anterior part of the caudal fin fork), were measured and registered at four time points: March 2018 (day 0), May 2018 (day 67), March 2019 (day 369) and September 2019 (day 537) (Table 2). Condition factor (κ factor calculated by Fulton's conditioning factor (Schreck & Moyle, 1990) was calculated at each time point, and specific growth rate (SGR calculated by (e^q - 1) 100 (Houde & Schekter, 1981), where $q = (W_2/W_1)^{1/(t2-t1)}$ and W_1 and W_2 are the weights at the first (t_1) and last (t_2) timepoint, was calculated at day 67, 369 and 537. Heart size was measured by weight (grams) at day 369 and 537, and further calculated based on cardio somatic index (CSI (%) = 100 (heart mass/BM)) at day 537. Mature fish were identified by their secondary sexual characteristics and large gonads. Sexual maturation was measured at day 537 and referred to as % matured individuals.

Macroscopic evaluation and sampling of white skeletal muscle discoloration were conducted at three time points: Day 67 ($n = 20$), day 369 ($n = 804$) and day 537 ($n = 627$). All samplings included fish from all experimental groups (Table 2). Macroscopic evaluation was performed using the scoring system developed by Mowi, grading RFC and MFC from grade 1 (small change with faint discoloration) to grade 3 (large change with severe discoloration) (Bjørgen et al., 2019). Based on discoloration, muscle tissue was sampled, labelled and transferred to 10% formalin for preservation and fixation for 3 to 10 days, in preparation for histological investigations.

2.4 | Pathogen detection

For detection of PRV-1, samples of spleen were collected and transferred to RNAlater for PCR examination conducted by PatoGen AS (Ålesund, Norway), with analyses accredited and validated to ISO7025 standards. Samples with a Ct-value lower than 37.0 was defined as positive. At day 67, the spleen was harvested from all 20 individuals (Table 3). At day 369, 46 individuals were tested for the presence of PRV-1, while 15 individuals were tested at day 537.

2.5 | Statistical investigations

To investigate a possible association between experimental groups and the presence of RFC and MFC, and between experimental groups and the grade of RFC/MFC, a Chi-squared test of independence was performed at day 369 and 537. A Kruskal-Wallis test was performed to analyse the difference in heart sizes (g) and body weight (g) across experimental groups at day 537. A Pearson correlation test was performed to assess the linear relationship between heart weight (g) and body weight (g). Logistic regression was used to analyse the relationship between heart size and the presence of RFC/MFC, and CSI and the presence of RFC/MFC, for each experimental group at day 537. *p*-values <0.05 were regarded as statistically significant. All data was analysed using StataCorp. 2019 (Stata Statistical Software: Release 16: StataCorp LLC). The data with variable descriptions are available at DOI: 10.18710/BXFHQ1 (DataverseNO).

TABLE 2 Parameters measured at day 0, 67, 369 and 537.

Day 0				
Parameter	Etne × Etne (n = 448)	Hybrid1 (n = 456)	Hybrid2 (n = 465)	Mowi × Mowi (n = 458)
Body weight (g)	46.15 (19.25)	78.93 (23.07)	77.98 (20.83)	131.19 (35.21)
Length (cm)	15.24 (2.37)	18.63 (1.93)	18.48 (1.68)	22.12 (2.26)
Condition factor (κ)	1.19 (0.09)	1.18 (0.07)	1.20 (0.07)	1.18 (0.07)
Day 67				
Parameter	**Etne × Etne (n = 428)**	**Hybrid1 (n = 441)**	**Hybrid2 (n = 458)**	**Mowi × Mowi (n = 453)**
Body weight (g)	55.35 (21.10)	92.44 (25.19)	93.51 (25.34)	152.40 (40.71)
Length (cm)	16.40 (2.43)	19.91 (1.83)	19.79 (1.83)	23.53 (2.22)
Condition factor (κ)	1.17 (0.10)	1.14 (0.07)	1.17 (0.07)	1.14 (0.06)
SGR (% day)	0.24 (0.20)	0.24 (0.13)	0.28 (0.11)	0.23 (0.09)
RFC (% of origin)[a]	0	0	0	0
MFC (% of origin)[a]	0	0	0	0
Day 369				
Parameter	**Etne × Etne (n = 151)**	**Hybrid1 (n = 208)**	**Hybrid2 (n = 215)**	**Mowi × Mowi (n = 230)**
Body weight (g)	1367. 06 (592.01)	2526.63 (598.01)	2152.56 (628.73)	3392.53 (624.60)
Length (cm)	47.30 (6.56)	57.19 (3.97)	54.78 (5.07)	62.65 (3.30)
Condition factor (κ)	1.19 (0.11)	1.31 (0.12)	1.26 (0.10)	1.36 (0.10)
SGR (% day)	1.02 (0.15)	1.10 (0.09)	1.04 (0.09)	1.04 (0.07)
Heart weight (g)[b]	1.86 (0.68)	3.56 (0.69)	2.76 (0.69)	4.74 (0.89)
RFC (% of origin)	7	5	5	3
MFC (% of origin)	27	26	28	28
Day 537				
Parameter	**Etne × Etne (n = 137)**	**Hybrid1 (n = 165)**	**Hybrid2 (n = 178)**	**Mowi × Mowi (n = 147)**
Body weight (g)	2392.72 (1180.71)	4547.90 (1213.77)	3944.81 (1142.13)	5932.85 (1367.817)
Length (cm)	57.94 (8.65)	71.21 (6.05)	68.52 (6.30)	77. 35 (6.24)
Condition factor (κ)	1.13 (0.14)	1.22 (0.13)	1.18 (0.11)	1.25 (0.12)
SGR (% day)	0.76 (0.08)	0.83 (0.06)	0.79 (0.07)	0.78 (0.07)
Heart weight (g)	3.70 (1.78)	6.55 (1.93)	5.97 (1.67)	8.46 (2.12)
CSI (%)	0.15 (0.02)	0.14 (0.02)	0.15 (0.02)	0.14 (0.02)
RFC (% of origin)	1	1	1	0.6
MFC (% of origin)	28	20	32	23

Note: n = observations. The numbers are mean (S.D) or frequency within experimental group (%). Hybrid1 = Etne × Mowi, Hybrid2 = Mowi × Etne.
Abbreviations: CSI, cardio somatic index; MFC, melanized focal changes; RFC, red focal changes; SGR, Specific growth rate.
[a]At day 67, 20 fish were sampled for macroscopic evaluation of fillet (EE = 8, Hybrid1 = 1, Hybrid2 = 2, MM = 9.
[b]At day 369, heart weight was measured in 95 fish (EE = 17, Hybrid1 = 32, Hybrid2 = 25, MM = 21).

2.6 | Histological investigations

Histological investigations of all experimental groups were conducted at day 67, 369 and 537 (Table 3) and included transverse sections of white skeletal muscle. The sections included both RFC, MFC and controls (no discoloration). Prior to sectioning, the samples were cropped to the preferred size for block preparation and paraffin embedding was conducted according to standard procedures. The sections were cut to a thickness of 2 μm, transferred to glass slides and incubated in 37°C for 36–48 h. Following deparaffinization in xylene the sections were rehydrated in alcohol baths and stained following a haematoxylin and eosin staining protocol. Fontana Masson stain was conducted to identify melanin pigment (Bancroft & Gamble, 2008). By using a developed classification system on histological changes in melanized white skeletal muscle (Bjørgen et al., 2019) the histological sections were categorized from 1 to 9. Additional changes were noted.

To acquire an adequate number of samples from each experimental group, both macroscopic and histological investigations of samples from day 67 were conducted with knowledge of genetic

TABLE 3 Number of samples (*n*) in each experimental group selected for histological investigations of skeletal muscle and Rt-qPCR analyses for the presence of PRV-1 in spleen.

	Etne × Etne (*n*)	Hybrid1 (*n*)	Hybrid2 (*n*)	Mowi × Mowi (n)
Day 67				
Histology	8	1	2	9
Rt-qPCR	8	1	2	9
Day 369				
Histology	9	16	15	8
Rt-qPCR	9	15	14	8
Day 537				
Histology	17	15	21	15
Rt-qPCR	0	4	5	6

Note: Hybrid1 = Etne × Mowi, Hybrid2 = Mowi × Etne.

origin. However, at 369 and 537, the analyses were conducted blinded to the investigators with respect to genetic origin. The experimental group was revealed after the material had been analysed and classified.

3 | RESULTS

3.1 | Macroscopic results

3.1.1 | Growth parameters

At day 537, farmed salmon (Mowi x Mowi) showed the highest exponential increase in body weight of all experimental groups with a mean weight at 5932 g (±1367). Mean weight of Hybrid1 (Etne x Mowi) were 4547 g (±1213) and Hybrid2 (Mowi x Etne) 3944 g (±1142). Wild salmon (Etne x Etne) had a mean weight of 2392 g (±1180). Length measurements showed results corresponding to the weight registrations: Mean length of farmed salmon was 77 cm (±6), Hybrid1 was 71 cm (±6) and 68 cm (±6) for Hybrid2. Wild salmon mean length was 58 cm (±8) at day 537. At day 537, significant differences were found in mean body weight between all experimental groups (χ^2 (3) = 306,683, $p < .05$).

Using body weight and length, body condition (κ factor) was calculated. At the trial starting point, wild salmon mean κ factor was highest, followed by Hybrid2, Hybrid1 and farmed salmon. At day 369, the results were reversed, as farmed salmon showed highest mean κ factor at 1.36 (±0.10), Hybrid1 at 1.32 (±0.12), Hybrid2 at 1.26 (±0.10) and wild salmon at 1.19 (±0.11). At day 537, all groups had a decline in body condition: Farmed salmon at 1.25 (0.12), Hybrid1 at 1.22 (±0.16), Hybrid2 at 1.18 (±0.11) and wild salmon at 1.13 (±0.14). SGR was calculated as percentage increase of growth per day. At day 369, mean SGR of wild salmon was 1.02 (±0.15), Hybrid1 was 1.10 (±0.09), Hybrid2 was 1.04 (±0.09) and farmed salmon was 1.04 (±0.07). At day 537, there was a decline in SGR within all experimental groups: Wild salmon: 0.76 (±0.08), Hybrid1: 0.83 (±0.06), Hybrid2: 0.79 (±0.07) and farmed salmon: 0.78 (±0.07).

3.1.2 | Heart size and maturation

At day 369 and 537, heart size was evaluated by weight (g). Mean heart weight (g) was lowest in wild salmon with 3.70 g (±1.78). Hybrid1 had a mean heart weight at 6.55 g (±1.93), and Hybrid2 was 5.97 g (±1.67). Farmed salmon had a mean heart weight at 8.46 g (±2.12). At day 537, cardio somatic index was calculated (CSI, % of body weight) (Frisk et al., 2020) (Table 2). Salmon of wild origin had the highest mean CSI at 0.158 (±0.02) followed by Hybrid2 at 0.154 (±0.02). Hybrid1 had a mean CSI of 0.145 (±0.02) and wild salmon had a CSI of 0.144 (±0.02). At day 537, significant differences were found in mean heart weight between all experimental groups (χ^2 (3) = 264.088, $p < .05$). There was a positive correlation between heart weight and body weight ($r = 0.9178$, $p < .05$). The logistic regression model conducted with RFC/MFC as response variable and experimental groups and heart weight (g) as explanatory variable/associated variables showed that none of the variables were significant with OR 1.07 ($p = .485$, 95% CI [0.88; 1.31]) and 0.95 ($p = .324$, 95% CI [0.87; 1.04]), respectively. The result was also not significant when using experimental groups and CSI (%) as explanatory variable/associated variables with OR 1.05 ($p = .058$, CI [0.86; 1.19]) and 1.98 ($p = .849$, CI [0.00; 2342.70], respectively.

Maturation was also evaluated at day 537, with the highest percentage registered in the hybrid groups, Hybrid1 (Etne x Mowi) 31% and Hybrid2 (Mowi x Etne) 28%. Wild salmon had a maturation percentage of 25% while the farmed salmon group had the lowest degree of maturation of only 15%.

3.1.3 | Red and melanized focal changes (RFC/MFC)

Macroscopic evaluation was conducted at day 67, 369 and 537. At day 67, no RFC or MFC were detected. At day 369, discoloration was detected in 264 of 804 fish, herein 41 RFC and 223 MFC. Thirteen fish had both RFC and MFC. At day 537, discoloration was detected in 171 of 627 fish, herein seven RFC and 164 MFC. Two fish had both RFC and MFC. In general, the prevalence of RFC and MFC increased after seawater transfer (Figure 1). The prevalence of discoloration showed a minor decrease from day 369 to day 537. The prevalence (%) and distribution of RFC and MFC in the different experimental groups are shown in Table 2 and Figure 2. There was no statistically significant association in the presence of RFC and MFC between the experimental groups at day 369 (χ^2 ($df = 9$, $n = 804$) = 5.4320, $p = .795$) or day 537 (χ^2 ($df = 9$, $n = 627$) = 6.9193, $p = .646$). By visual examination, all samples were scored by the Mowi classification system, grading the RFC and MFC from 1 to 3. In all experimental groups, grade 1 was the most frequent score, followed by grade 2. Grade 3 was the least observed grade in all experimental groups. At both day 369 and 537 there was no statistically significant

association between RFC grade and experimental groups (χ^2 (df = 9, n = 804) = 12.1404, p = .206, χ^2 (df = 9, n = 627) = 10.0244, p = .349), or MFC grade and experimental groups (χ^2 (df = 9, n = 804) = 9.6834, p = .377, χ^2 (df = 9, n = 627) = 16.4981, p = .057).

3.2 | PRV-1 (RT-qPCR)

At day 67, PRV-1 was not detected in any fish (n = 20). At day 369, 45 of 46 samples tested positive for PRV-1 (mean Ct-value 27.03). At day 537, 15 of 15 samples tested positive for PRV-1 (mean Ct-value 32.19).

3.3 | Histological investigations

3.3.1 | Day 67

At day 67, all experimental groups were represented within the obtained 20 samples. In all samples, most myocytes presented no pathological changes. Melano-macrophages were predominantly confined within the connective tissue adjacent to blood vessels, but also occasionally interspersed between adipocytes in the zone between red and white muscle tissue (Figure 3a–d). Melanin pigment was confirmed with Fontana Masson stain (Figure 3e,f). Both locations of melano-macrophages were present in all experimental groups. No additional inflammatory changes were revealed in any changes in any of the experimental groups.

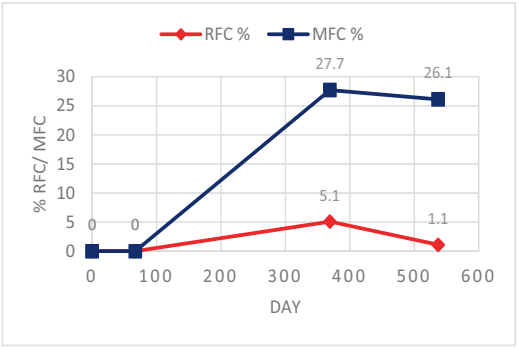

FIGURE 1 Prevalence of red (RFC) and melanized (MFC) focal changes at each sampling point (day 67, 369 and 537).

3.3.2 | Day 369 and 537

At day 369, 46 samples were evaluated by histological investigations, herein eight RFC (Etne x Etne: n = 1, Hybrid1: n = 3, Hybrid2: n = 2, Mowi x Mowi: n = 2) and 30 MFC. Eight samples had no macroscopic discoloration. At day 537, 68 samples were selected for histology, herein five RFC (Etne x Etne: n = 0, Hybrid1: n = 3, Hybrid2: n = 2, Mowi x Mowi: n = 1) and 61 MFC. Two samples had no macroscopic discoloration.

Histological findings of RFC at both sampling points (day 369 and 537) in each experimental group were characterized by haemorrhage and necrotic myocytes, coinciding with the changes described by Bjørgen et al., 2019. Extravascular erythrocytes were seen between myocytes and interspersed in adipose tissue both between necrotic myocytes (Figure 4a) and in the myosepta (Figure 4b). In more progressed changes, melano-macrophages were found scattered in the affected tissue, however, not as abundant as in MFC. Here, vacuoles, inflammatory cells and fibroblasts were also present (Figure 4c).

The MFC largely coincided with the histological descriptions presented by Bjørgen et al., 2019. This included melano-macrophages interspersed between intact myocytes (category 2), infiltration in the muscle tissue of melano-macrophages and other inflammatory cells, with fibrosis (category 6) and diffuse and nodular granulomatous inflammation and presence of myocyte regeneration (category 8 and 9). Both low- and high-grade categories were observed in all experimental groups. In addition, some changes not represented in the established category system were observed. These changes were seen in all experimental groups and included melano-macrophages in the adipose tissue in the myosepta and perimysium, and various types of vacuoles and/or granulomas. At day 537, histological examination revealed prominent changes in MFC characterized

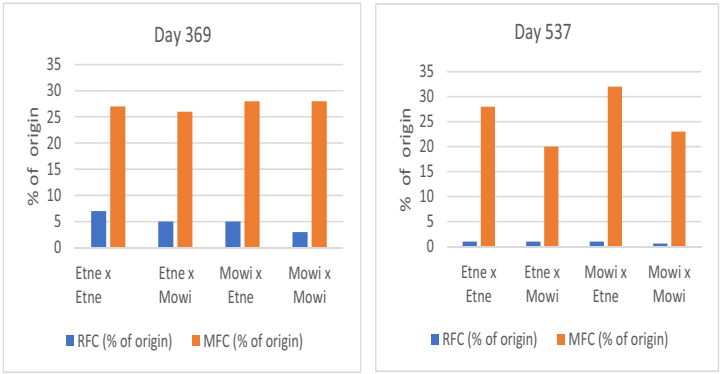

FIGURE 2 Distribution of red (RFC) and melanized (MFC) focal changes (%) between experimental groups at day 369 (a) and day 537 (b).

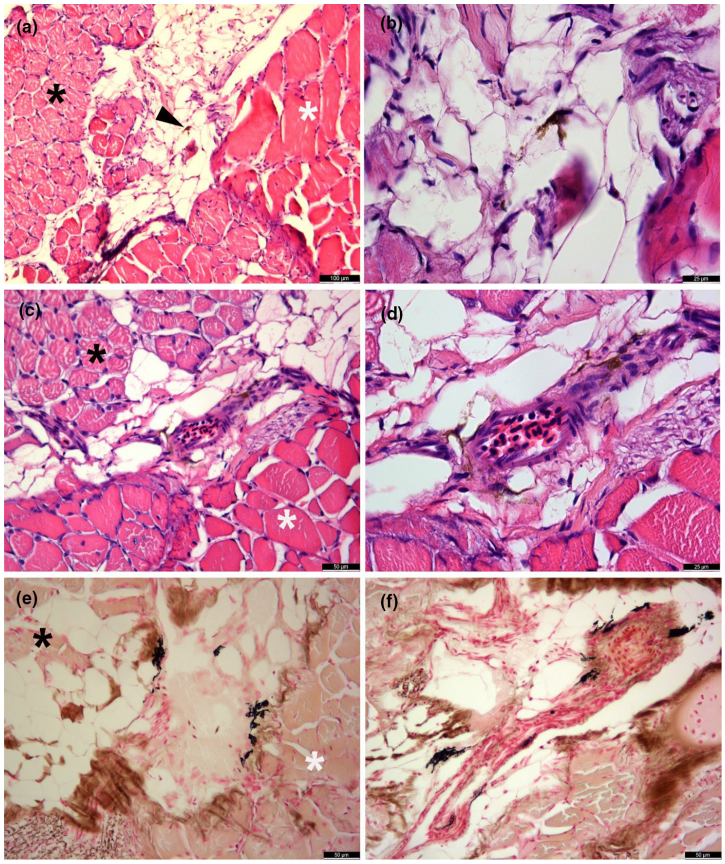

FIGURE 3 Histological changes in skeletal muscle in fish sampled at day 67. No macroscopic discoloration observed. (a) Farmed salmon (Mowi × Mowi). Area between red (black asterisk) and white (white asterisk) skeletal muscle. Melano-macrophage (arrowhead) interspersed between adipocytes. HE stain, scale bar = 50 μm. (b) Larger magnification of area in (a) with melano-macrophage in adipose tissue. HE stain, scale bar = 25 μm. (c) Wild salmon (Etne × Etne). Melano-macrophages present in the connective tissue of a blood vessel. HE stain, scale bar = 50 μm. (d) Larger magnification of area in (c) with extravascular melano-macrophages. HE stain, scale bar = 25 μm. (e) Wild salmon (Etne × Etne). Positive melanin staining in area between red (black asterisk) and white (white asterisk) skeletal muscle. Fontana Masson stain, scale bar = 50 μm. (f) Farmed salmon (Mowi × Mowi). Positive staining for melanin adjacent to blood vessel. Fontana Masson stain, scale bar = 50 μm.

by the presence of vacuoles and melano-macrophages, as well as granulomas in putative various stages. These stages included well-organized granulomas (Figure 5a), granulomas with central vacuoles (Figure 5b), enlarged central vacuole with fewer surrounding macrophages (Figure 5c), and vacuoles with few surrounding immune cells, except for elongated melano-macrophages lining the rim of the vacuole (Figure 5d). Using Fontana Masson stain, melanin pigment was confirmed in these different stages (Figure 5e,f).

4 | DISCUSSION

This study aimed to assess the genetic influence on the occurrence of RFC and MFC in Atlantic salmon. Individuals from wild, farmed and hybrid origins were reared under identical conditions and sampled before and after transfer to sea and followed until reaching slaughter weight. RFC and MFC were detected after sea cage transfer, and their prevalence increased throughout the production period, although a minor decrease was registered at slaughter. Importantly, no significant difference in the prevalence of RFC and MFC was found between the four experimental groups.

In contrast, our study revealed significant differences in growth parameters between farmed and wild salmon, with the hybrids displaying intermediate characteristics. These results align with previous reports investigating wild, hybrid and farmed fish kept in common conditions (Glover et al., 2009; Perry et al., 2021; Thodesen et al., 1999). Specific growth rate (SGR) and body condition (κ factor) are commonly used metrics in salmonid aquaculture to assess growth and quality, respectively. A higher κ factor value in general indicates better growth and quality (Barnham & Baxter, 1998; Stien et al., 2013) and is correlated with increased fat content of the fish (Einen et al., 1998; Hamre et al., 2004). However, an increased κ factor has been observed in fish with deformed vertebra (Hansen et al., 2010). The κ factor also varies during the production cycle (Mørkøre & Rørvik, 2001) and is thus influenced by multiple factors. The observed decline in SGR and k factor at day 537 can be attributed to variations in day length (photoperiod), as both photoperiod and temperature have been shown to influence these parameters in previous studies (Fjelldal et al., 2009; Nordgarden et al., 2003). It is important to note that SGR tends to decrease with increasing fish size (Jobling, 2010).

In our study, we found significant differences in heart size (g) among all experimental groups. However, we observed a high correlation between heart size and body size. A previous study examining farmed, wild and hybrid Atlantic salmon kept in common rearing conditions did not detect any differences in heart size and

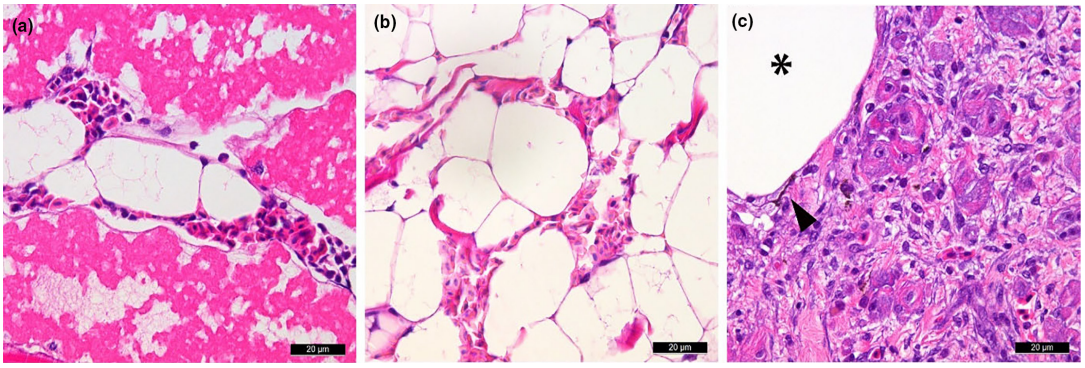

FIGURE 4 Histological changes in red focal changes (RFC).HE stain. Hybrid1 (Etne × MowiOWI), sampled at day 369 selected for illustration. (a) Erythrocytes and inflammatory cells in adipose tissue between necrotic white skeletal myocytes. Scale bar = 20 µm. (b) Area with haemorrhage in adipose tissue in myosepta. Scale bar = 20 µm. (c) Area in myotome with myocyte necrosis, and presence of erythrocytes, fibroblasts and inflammatory cells. Melano-macrophages are also present (arrowhead). Note the large vacuole (asterisk). Scale bar = 20 µm.

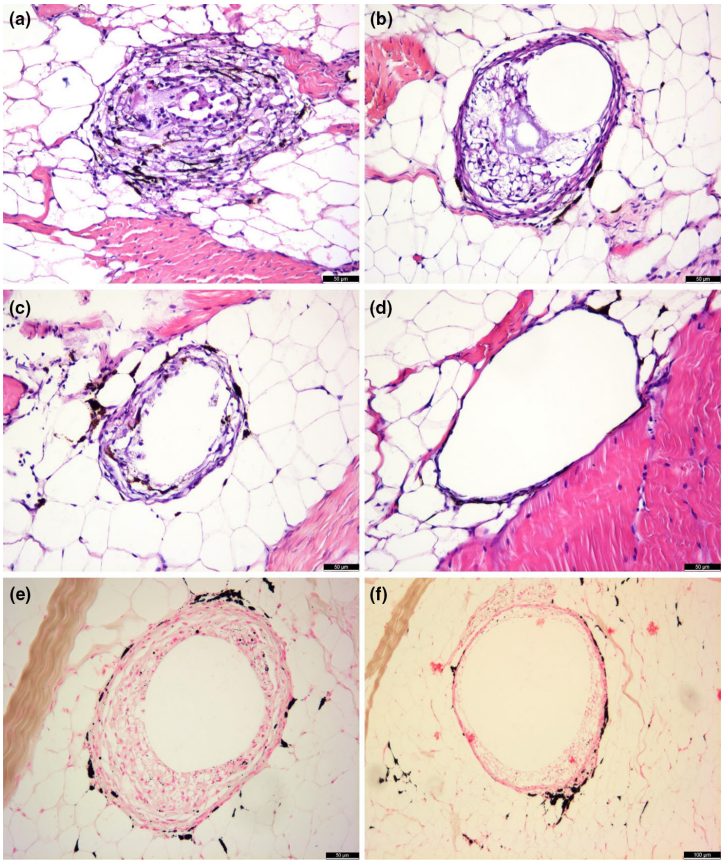

FIGURE 5 Histological changes seen in MFC at day 537. (a) Hybrid1 (Etne × Mowi). Well-organized, cell-rich granuloma, with elongated melano-macrophages and macrophage-like cells. HE stain, scale bar = 50 µm. (b) Hybrid2 (Mowi × Etne). Granuloma with central vacuole and surrounding melano-macrophages and macrophage-like cells. HE stain, scale bar = 50 µm. (c) Hybrid2 (Mowi × Etne). Larger central vacuole, surrounded by elongated melano-macrophages and less abundant macrophage-like cells. HE stain, scale bar = 50 µm. (d) Wild salmon (Etne × Etne). Vacuole surrounded by elongated melano-macrophages, deprived of macrophage-like cells. HE stain, scale bar = 50 µm. (e) Hybrid2 (Mowi × Etne). Large vacuole with elongated cells staining positive for melanin. Fontana Masson stain, scale bar = 50 µm. (f) Hybrid2 (Mowi × Etne). Elongated cells staining positive for melanin surrounding a vacuole and in adjacent adipose tissue. Fontana Masson stain, scale bar = 100 µm.

morphology when accounting for body size (Perry et al., 2020). These results suggest that the altered heart morphology and weight observed in farmed salmon compared to their wild counterparts (Poppe et al., 2003) are likely attributable to environmental factors, such as altered activity levels (Gamperl & Farrell, 2004) and hatchery protocols (Brijs et al., 2020; Frisk et al., 2020).

It has been suggested that cardiac malformations may lead to decreased oxygen transport due to reduced cardiac output (Frisk et al., 2020; Johansen et al., 2017; Poppe et al., 2003). This could potentially result in hypoxia in the skeletal muscle, leading to myocyte necrosis and subsequent local inflammation. While we did not specifically investigate cardiac morphology in this study, we found

no significant relationship between heart weight and CSI and the presence of RFC/MFC. Therefore, the development of the focal skeletal muscle inflammation does not appear to be linked to these heart parameters.

Macroscopically, RFC and MFC were registered and scored from 1 to 3. Visual examination of the fillets at three different timepoints revealed the absence of RFC and MFC before seawater transfer, with an increased prevalence observed after transfer to sea. This pattern of increasing prevalence after sea transfer is consistent with the findings of Bjørgen et al., 2019. However, our study diverges from their results as we observed a decline in MFC at the point of slaughter. In chronic inflammatory conditions, the rate of inflammation and repair depends on various factors, including the size of the changes, the removal of any persisting agent and the regenerative capacity of the tissue involved (Zachary, 2017). It is important to note that teleosts are poikilothermic vertebrates that adjust to the surrounding temperature. Thus, sea water plays a significant role in influencing the rate inflammation and repair, even during chronic granulomatous inflammation (Le Morvan et al., 1998; Roberts, 2012; Timur et al., 1977). Our study ended in August, during a period with relatively high seawater temperature (C°), whereas the final sampling in the study of Bjørgen et al., 2019 took place in December when seawater temperatures were lower. Therefore, the decline of MFC observed in our study may be temperature-related, with higher temperatures providing a more favourable environment for healing and tissue repair.

In the statistical analysis, we found no significant differences in the distribution and grade of RFC and MFC among the experimental groups. This indicates that the dissimilar prevalence of RFC/MFC in farmed and wild salmon is not primarily influenced by genetic (internal) factors, but rather by external factors, such as nutrition, density, milieu and handling practices. During the seawater phase of our study, fish handling was minimized. The attached sea lice were manually removed, eliminating the need for chemical or mechanical treatments. No other infections were treated throughout the trial. However, the presence of PRV-1 was detected through Rt-qPCR analyses of spleen at day 369 and 537. Previous reports have confirmed that Atlantic salmon may be infected with PRV-1 without presenting clinical signs of disease, that is heart and skeletal muscle inflammation (Wessel et al., 2017; Wessel et al., 2020). Our results show that PRV-1 was present in the spleen of the majority of the fish in the seawater phase, thus the local presence of PRV-1 within RFC and MFC was not investigated. However, the link between PRV-1 infection and MFC has been addressed in previous work, indicating the virus to be a chronic trigger of inflammation due to persistent presence and replication within the most severe, granulomatous changes (Bjørgen et al., 2015, 2020). The presence of PRV-1 in samples obtained during the seawater phase, combined with the marked increase of RFC and MFC at sea, are in line with the previous associations that have been reported between PRV-1 infection and RFC/MFC.

The density of fish in the seawater cages (0.7 kg m^3) was relatively low compared to the maximum acceptable density in standard production (25 kg/m^3) (Lovdata, 2008). Therefore, external trauma resulting from handling and installations during the sea cage stage is unlikely to have played a significant role in the development of RFC and MFC in our study. However, it is important to note that the overall prevalence of MFC in the seawater phase of our study was approximately 25%, which aligns with previous reports from commercial production. This evident increase in prevalence suggests that the underlying cause of these changes may occur immediately before or after sea transfer, potentially attributed to physiological, infectious, nutritional, or other production-related factors.

Following macroscopical evaluation, we conducted histological investigations to further examine the focal discolorations observed in each experimental group. As melano-macrophages may contain several pigments, such as melanin, lipofuscin and hemosiderin (Agius, 1985; Agius & Roberts, 2003) the presence of melanin pigment was confirmed through Fontana Masson stain. At day 67, prior to seawater transfer, no macroscopic changes were visible. However, samples containing both red and white skeletal muscle were sampled to investigate potential histological changes. In these samples, scattered melano-macrophages were detected in close proximity to vessels across all experimental groups. While melano-macrophages and melanin are typically abundant in lymphoid organs like the head kidney and spleen (Thorsen et al., 2006), they can also be found in a perivascular location (Roberts, 1975), particularly in farmed fish, as seen in cod (Cooper & Midling, 2007). At day 67, melano-macrophages were also observed interspersed in the adipose tissue between red and white skeletal muscle in half of the samples, with all experimental groups represented. The myosepta between white muscle myotomes was difficult to evaluate at this time point, likely due lower fat composition in the feed at this point of production compared to later phases (FAO, 2023). As a result, the presence of melano-macrophages in myoseptal adipose tissue could not be evaluated. In a previous study, RFC were observed in freshwater smolts, but not MFC (Jiménez-Guerrero et al., 2022). In addition, histological investigations were not performed in their study, thus the presence of inflammatory cells including melano-macrophages was not addressed. The dynamics of melano-macrophages in inflammatory conditions during the freshwater stage remain largely unknown and should be subject for future investigations.

The observed focal changes at both sampling points in sea water (day 369 and 537) aligned with the histological descriptions of RFC and MFC provided by Bjørgen et al., 2019 and Bjørgen et al., 2020. RFC were present in all experimental groups and exhibited similar characteristics. The histopathological features of MFC generally corresponded to the classification used in Bjørgen et al., 2019, with a few exceptions. One notable finding was the presence of melano-macrophages in the adipose tissue within the myosepta and perimysium, with no other changes detected. The presence of melano-macrophages in the adipose tissue could result from recruitment and actions of inflammatory cells caused by a previous haemorrhage and acute inflammation, as seen in RFC. Additionally, an inflammatory state within the adipose tissue induced by adipocytes

themselves could lead to recruitment of inflammatory cells. Reports have indicated an increase in recruited macrophages within adipose tissue of obese humans due to elevated levels of pro-inflammatory mediators (Li et al., 2023; Page et al., 2011). Inflammation of adipose tissue can potentially be induced by hypoxia resulting from adipocytes hypertrophy (Trayhurn et al., 2008). Interestingly, an increase of adipocyte size has been observed in fish-fed vegetable oils, which are commonly included in standard commercial diets due to high costs of fish oil (Cruz-Garcia et al., 2011; Tacon & Metian, 2008). Importantly, a lower dietary content of fish oils, which also leads to lower levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been associated with a higher prevalence of MFC (Lufti et al., 2023; Sissener et al., 2016). The histopathological occurrence of fat in RFC and MFC has to a certain extent been addressed in previous reports. In these reports, the focus was not solely on the location of the granulomas in relation to adipose tissue, but rather the more general role of fat in growth and feeding composition, and the possible correlation between a high-fat content and RFC/MFC (Mørkøre et al., 2016). The granulomas in previous reports were located in relation to white, but not red, skeletal muscle, however, with varying degree of adipocytes present. The adipocytes are suggested to play an active role in the inflammation, as seen in some conditions in mammals (Kawai et al., 2021), however, an initial damage to the adipose tissue cannot be ruled out. In the rare condition of melanized red skeletal muscle, granulomas were not detected (Brimsholm et al., 2022).

The occurrence of granulomas and vacuoles in MFC observed at day 537 has previously been documented in both vaccinated and non-vaccinated Atlantic salmon (Berg et al., 2007; Koppang et al., 2005; Larsen et al., 2012, 2014). In these studies, granulomas were described as well-organized structures containing macrophages, that is melano-macrophages, multinucleated giant cells and epithelioid-like cells. The extracellular vacuoles were filled with erythrocytes and macrophage-like cells, or containing lipids, or they were empty. Vacuoles were also observed centrally within organized granulomas. Our findings align with these previous reports on different stages of granulomas in MFC. It is plausible to suggest that these putative different stages may represent a healing process, culminating in the formation of relatively large vacuoles surrounded by melano-macrophages. Granulomas are commonly observed in chronic inflammation in fish (Roberts, 2012), and their development and characteristics can vary depending on the underlying cause and fish species (Balouet & Laurencin, 1986). Notably, large vacuoles have also been observed in advanced RFC without the presence of granulomas (Bjørgen et al., 2020), indicating that the development of vacuoles without pre-existing granulomas is also a possibility.

In conclusion, our study demonstrates the occurrence of RFC and MFC in genetically wild Atlantic salmon, selectively farmed Atlantic salmon and hybrid Atlantic salmon when subjected to identical rearing conditions. The prevalence of RFC/MFC in these different fish groups indicates that environmental factors associated with the rearing conditions play a significant role in their development. Genetic factors, on the other hand, do not seem to be the driving force behind the occurrence of RFC and MFC. These findings highlight the importance of considering and optimizing rearing conditions to minimize the prevalence of these lesions in farmed Atlantic salmon.

AUTHOR CONTRIBUTIONS

Håvard Bjørgen: Conceptualization; methodology; software; investigation; supervision; project administration; writing – review and editing; writing – original draft; visualization; funding acquisition; formal analysis; validation. **Malin Brimsholm:** Software; validation; investigation; formal analysis; visualization; writing – original draft; writing – review and editing; project administration. **Per Fjelldal:** Writing – review and editing; conceptualization; methodology; funding acquisition; resources; project administration. **Monica Solberg:** Methodology; investigation; writing – review and editing. **Erling Olaf Koppang:** Conceptualization; methodology; investigation; formal analysis; writing – original draft; writing – review and editing; validation; funding acquisition; resources; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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IV

Red and melanized focal changes in the white skeletal muscle of farmed rainbow trout (*Oncorhynchus mykiss)*

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Running page head: Melanized changes in farmed rainbow trout

Abstract

Fillet discoloration by red and melanized focal changes (RFC and MFC) is common in farmed Atlantic salmon (*Salmo salar*). In farmed rainbow trout (*Oncorhynchus mykiss*), similar changes have been noted but their prevalence and histological characteristics have not been investigated. Thus, we conducted a study from three different farm sites in Norway encompassing 1293 rainbow trout, all examined at the time of slaughter. Both macroscopic and histological assessments of the changes were performed. RT-qPCR analyses and *in situ* hybridization (ISH) were used to detect the presence and location, respectively, of potential viruses. RFC were identified in only one fish, while the prevalence of MFC ranged from 1.46% to 6.47%. The changes were predominantly localized in the cranioventral region of the fillet. Histological examinations unveiled necrotic myocytes, fibrosis, and regeneration of myocytes. Melano-macrophages were found in the affected areas and in myoseptal adipose tissue. Organized granulomas were observed in only one fish. Notably, the presence of inflammatory cells, including melano-macrophages, appeared lower compared to what has been previously documented in Atlantic salmon MFC. Instead, fibrosis and regeneration dominated. RT-qPCR and ISH revealed the presence of piscine orthoreovirus-1 (PRV-1) and salmonid alphavirus (SAV) in skeletal muscle. However, these viruses were not consistently associated with lesioned areas, contrasting previous findings in Atlantic salmon. In conclusion, the rainbow trout develops MFC of a different character than farmed Atlantic salmon, and we speculate if the observed pathological differences are contributing to their reduced occurrence in farmed rainbow trout.

Keywords: Fillet; Melanin; Melano-macrophage; Piscine orthoreovirus 1; Rainbow trout; Salmonid alphavirus; Skeletal muscle

1. Introduction

With an approximate prevalence of 20% in the Norwegian Atlantic salmon (*Salmo salar*) production, the occurrence of melanized focal changes (MFC) in the white skeletal muscle (or the fillet) poses a significant quality and economic concern (Mørkøre et al., 2015). The discoloration is attributed to the presence of melano-macrophages, a pigment-producing leukocyte population unique to ectothermic vertebrates (Sichel, Scalia, Mondio, & Corsaro, 1997). Melano-macrophages can be found in lymphoid organs and inflamed tissues of fish (Agius, 1981; Larsen et al., 2012; Thorsen, Høyheim, & Koppang, 2006). While severe melanized white muscle changes in salmon have been associated with *Piscine orthoreovirus* 1 (PRV-1) infection, they can also occur independently of virus presence (Bjørgen et al., 2019; Bjørgen et al., 2015). The MFC are preceded by focal hemorrhagic lesions (RFC – red focal changes) that may progress into chronic inflammatory changes containing melano-macrophages over time (Bjørgen et al. 2019). In less severe cases, various histological changes may be observed, often with melano-macrophages dispersed among seemingly unaffected myocytes. In more severe forms, granulomatous inflammation in conjunction with replicating PRV-1 within melanized granulomas has been described (Bjørgen et al., 2019; Bjørgen et al., 2015). The initiating causes of the RFC remain undetermined (Bjørgen et al. 2019).

MFC have also been noted in the rainbow trout (*Oncorhynchus mykiss*) by the fish farming industry, although no peer-reviewed publications have addressed this condition. According to the industry, the prevalence of MFC is negligible in this species, resulting in limited efforts to study the condition. Nonetheless, investigation of such changes in rainbow trout in comparison to similar changes in Atlantic salmon can provide new insight into the pathogenesis of the condition. It is noteworthy that the commercial production conditions of

rainbow trout, i.e., seawater locations, vaccination regimes, feed etc. are very similar to those of Atlantic salmon. The susceptibility of rainbow trout and Atlantic salmon to various viral infections varies, and this may be particularly important in this matter for viruses known to induce inflammation in skeletal muscles and being prevalent in aquaculture of salmonids in Norway, such as salmonid alphavirus (SAV) and PRV-1.

PRV-1- 3 and SAV 1-6 variants cross-infect a range of salmonid species and appear to cause species-specific disease dependent on virus genotype. PRV and SAV are often found to infect the same individual (Wiik-Nielsen, Alarcón, Bang Jensen, Haugland, & Mikalsen, 2016). PRV-1 infection is endemic in farmed Atlantic salmon after seawater transfer and often leads to outbreaks of heart- and skeletal muscle inflammation (HSMI), one of the most common viral diseases in Norwegian aquaculture (Olsen & Dahle, 2023). PRV-3 has occasionally caused outbreaks in rainbow trout freshwater facilities, characterized by both heart inflammation and anemia (Olsen, Hjortaas, Tengs, Hellberg, & Johansen, 2015). In contrast, PRV-1 has not been associated with disease in farmed Rainbow trout (Purcell et al., 2020), and PRV-3 does not lead to disease in Atlantic salmon (Malik et al., 2021c). Whereas Atlantic salmon are persistently infected by PRV-1, rainbow trout appear to be able to fight off both PRV variants after a period of infection (Hauge et al., 2017). Several salmonid Alphavirus (SAV) subtypes cause Pancreas Disease (PD) in farmed Atlantic salmon in Europe, a notifiable disease of high concern that also affects the skeletal muscle (Sindre, Patel, Olsen & Løkslett, 2023). A freshwater SAV-2 variant can cause Sleeping Disease (SD) in rainbow trout, but seawater SAV variants rarely cause serious disease in rainbow trout.

In the present study, we investigated focal melanized changes in three distinct populations of farmed rainbow trout. The production and environmental conditions were similar to those of farmed Atlantic salmon, and thus, the study was well-suited for species comparisons, including differences in susceptibility to various pathogens. Our results

revealed that the MFC in rainbow trout appear macroscopically similar to those in Atlantic salmon but have lower prevalence and show certain microscopical differences that seem characteristic for such changes in trout. We discuss possible explanations for these differing characteristics in farmed rainbow trout MFC and whether these variations may be attributed to inherent defense mechanisms or immune responses to viral infections that impact skeletal muscle.

2. Materials and Methods

2.1 Fish populations

Three marine farms with rainbow trout in Western Norway were selected for this study. Farm 1 was in Laksevika, Fram 2 in Tepstad, and Fram 3 in Djupestallen. The samples were obtained at slaughter during the spring of 2017 and were provided by Lerøy Vest AS/Sjøtroll Havbruk AS and Blom Fiskeoppdrett. Fish from Farm 1 were sampled on April 11^{th,} and 616 individuals sorted in the weight category 4-5 kg were investigated at the slaughter line. Farm 2 was sampled on April 18th, 417 individuals in the weight category 5-6 kg. Farm 3 was sampled on May 2nd, 260 individuals in weight category 4-5 kg. At slaughter, muscle abnormalities were registered according to anatomical location (Fig. 1) and severity of observed macroscopic changes (scale 1-8 according to Mørkøre et al. (2012) (Fig. 2).

2.2 Sampling for transcriptional (RT-qPCR) and histological analysis

To test the populations for the presence of PRV-1, PRV-3, and SAV, hearts and spleen were collected in RNAlater at the slaughter line (Table 1). To test RFC/MFC for virus presence, affected muscle tissue was collected in RNAlater. Control tissue, i.e., non-affected muscle from the corresponding fillet and hearts, were also collected. In addition, samples from the same hearts, affected muscle, and control muscle were collected on buffered formalin for histological analysis. For details, see Table 1.

2.3 Viral analysis by RT-qPCR

Samples in RNAlater were sent to PatoGen AS, Ålesund, Norway, for RT-qPCR analysis for PRV-1, PRV-3, and SAV. The RT-qPCR analyses are accredited and validated to ISO7025 standards. PatoGen AS does not disclose details of the purification method and PCR conditions due to issues related to competing patents.

2.4 Histological investigations

Samples from hearts and affected and non-affected skeletal muscle were transferred to buffered formalin, fixed for 48 to 72 h, and processed routinely through paraffin embedding for further examination. All selected samples were cut to 2 µm thick sections and incubated at 37 °C for 36-48 h on glass slides. Following deparaffinization in xylene and rehydration in alcohol baths, the sections were stained with hematoxylin and eosin (HE) stain according to protocol. To determine the histological changes in HE-stained sections, we used a histological classification system developed for Atlantic salmon (Bjørgen et al., 2019). Briefly, the following histological categories were applied: 1) no histological changes; 2) melano-macrophages in the endomysium between intact myocytes; 3) fibrosis in the endomysium without the presence of melano-macrophages; 4) fibrosis in the endomysium with presence of melano-macrophages; 5) melano-macrophages, fibrosis and presence of inflammatory cells in the endomysium; 6) organized scar tissue with presence of inflammatory cells 7) organized scar tissue with presence of inflammatory cells and melano-macrophages; 8) inactive granulomatous inflammation with presence of melano-macrophages ad organized granulomas and finally 9) active granulomatous inflammation with myocyte necrosis and myocyte re-generation and presence of melano-macrophages. Based on results from the HE stained sections, selected samples were stained using Van Gieson (VG) stain and Masson Trichrome stain (MTC) for the detection of collagen, and Fontana Masson stain for the detection of melanin.

141

142 2.5 *In situ* hybridization (ISH)

143 RNAscope 2.5 HD Assay-red (Advanced Cell Diagnostics, Newark, CA, USA) was used for
144 *in situ* hybridization (ISH) following the manufacturer's guidelines (Wang et al., 2012). In brief,
145 4 µm thick paraffin-embedded tissue sections were prepared from 11 fish. The samples were
146 selected based on their histological characteristics as evaluated in HE staining, combined with
147 detectable virus levels of PRV-1, PRV-3, or SAV (Cq values of <37) in the tissue. Due to high
148 Cq values and low prevalence of lesions, no samples from farm 1 were selected for ISH
149 analyses. From farm 2, three skeletal muscle samples with a Cq value ≤ 34,2 for PRV-1 were
150 selected. Two samples with organized granulomas from one fish were added, although these
151 samples had a Cq value ≥ 37 for all investigated viruses. Two heart samples were selected from
152 farm 2, one with a Cq value of 28,4 for PRV-3 and mild cardiac lesions, and one with a Cq
153 value of 24,92 for PRV-3 and 30,85 for SAV, with no pathological lesions detected in
154 histological examination. As farm 3 represented the lowest Cq values for SAV in skeletal
155 muscle, five skeletal muscle samples were selected for ISH detection of SAV in the tissue.

156 The slides were mounted on positively charged glass slides (Superfrost©; Mentzel) and air-
157 dried for a minimum of 24 h before being subjected to a 60 °C incubation for 90 min, followed
158 by two rounds of 5 min treatment with xylene and two rounds of 1 min treatment with 100%
159 ethanol for dewaxing. Subsequently, the samples underwent a 10 m treatment with hydrogen
160 peroxide to block endogenous peroxidase activity. The pretreatment included heat treatment in
161 RNAscope® Target Retrieval Reagent at 100 °C for 15 min, followed by a protease treatment
162 for 10 min at 40 °C to permeabilize the cells.

163 For hybridization, the slides were incubated with the target ZZ-probes (Table 2) for 2 h
164 at 40 °C. Signal amplification was obtained by sequentially incubating the slides with the six

amplification solutions provided in the assay kit. Signal detection was performed by incubating the slides with Fast Red chromogenic substrate for 10 min, and counterstaining was performed by immersing the slides in a 50% Gill's hematoxylin solution for 2 min. Finally, the samples were mounted using EcoMount (BioCare Medical, Pacheco, CA, USA). All probes were designed and produced by the manufacturer based on user-provided sequences and have been catalogued and made commercially available. Details regarding the probes and positive and negative control probes, including gene, target region, accession number, and the manufacturer's catalogue number, are available in Table 2. To serve as positive controls for all three viruses, infected tissue from Atlantic salmon was utilized (Supplementary data). In the case of PRV-1, this involved heart and skin samples; for PRV-3, spleen samples were employed, and for SAV, skeletal muscle samples were used. As a negative control, an RNAscope probe against the bacterial gene dapB was used on duplicates of the same sections from skeletal muscle to confirm the absence of background and non-specific cross-reactivity.

3. Results

3.1 Macroscopic observations

Farm 1 had a MFC prevalence of 1.46%. One individual also displayed a focal hemorrhage, deemed as an RFC. Farm 2 had a MFC prevalence of 6.47% and Farm 3 5.76%. No RFC were observed in these two farms. In all farms, no transient forms between RFC and MFC were observed. The MFC were primarily detected in the cranio-ventral region and the dorsal part of the fillet, and the changes were in general of a low severity. For details, see Table 3**.**

The severity of the changes followed the same trend in all three populations, with most changes graded 1, accounting for approximately two-thirds of the total number of registered changes. The second most prevalent change was grade 2, and the third was grade 4, while only two grade 8 changes were detected (Figure 2).

3.2 RT-qPCR

PRV-1 was detected in one muscle sample from Farm 1 with a Cq value of 34.7, and in two heart samples, with a mean Cq value of 32.6 (± 2.26) (Table 4). All affected muscle samples were negative for PRV-3 and SAV. In three heart samples, PRV-3 was detected with a mean Cq value of 31.41 (± 4.41). None of the heart samples were positive for multiple viruses.

All muscle samples from Farm 2 were negative for PRV-1 and PRV-3. For SAV, only two of 54 muscle samples were negative, and the mean Cq value in the remaining muscle samples was 26.9 (± 3.62). In the heart samples, SAV and PRV-3 were present in six out of ten samples, with a mean Cq value of 32.01 (± 3.06) and 30.94 (± 3.90), respectively. PRV-1 was not detected in any of the heart samples.

In Farm 3, 26 muscle samples were positive for PRV-1 with a mean Cq value of 33.4 (± 2.96). Nine out of 37 muscle samples were positive for SAV and had a mean Cq value of 34.8 (± 1.16). PRV-3 was not detected in any muscle samples but in four out of ten heart samples, with a mean Cq value of 32.7 (± 2,81). PRV-1 was detected in eight out of ten heart samples, with a mean Cq value of 27.0 (± 4,85). In four heart samples, both PRV-1 and PRV-3 were detected.

3.3 Histological analysis

Histological investigations were conducted on both affected skeletal muscle and control samples (Table 1. For detailed information, see supplementary data). Control samples, RT-qPCR negative for all viruses, showed no pathological changes.

When combining the macroscopic scoring with the histology, the histological characteristics within macroscopic score 1 varied from moderate myocyte necrosis to severe fibrosis. Melano-macrophages were observed in areas with fibrosis, surrounding vacuoles, and in adipose tissue in myosepta. The second most abundant macroscopic manifestation, score 2, was dominated with the same findings as in score 1 changes, but fibrosis could be more pronounced. In the more severe grades (score 4 and 8), the histological findings included necrosis, fibrosis, and vacuoles in the lesioned areas.

The histological changes observed in the single RFC included hemorrhage and necrosis in the skeletal muscle (Fig 3a). In MFC, only one fish contained organized granulomas (Fig 3b), while vacuoles were often abundant in the lesioned areas (Fig 3c). Melano-macrophages were observed surrounding the vacuoles and in the adipose tissue interspersed in the skeletal muscle and myosepta (Fig 3d). The Fontana Masson stain confirmed the presence of melanin (Fig 3e, f). In general, very scarce inflammatory infiltrates

were observed. Instead, there was a prominent presence of loose connective tissue dispersed between putatively regenerated myocytes seen as small myocytes in areas adjacent to intact skeletal muscle (Fig 4). Collagen fibers were detected by Van Gieson stain and Masson Trichrome stain, with both colors revealing similar results (Fig 4).

3.4 *In situ* hybridization (ISH)

Investigations for the presence of PRV-1 showed ISH signal in the selected sample having the lowest Cq value (Table 5). The detected signal was observed in relation to intact myocytes and not in degenerated or necrotic myocytes (Fig. 5 a). Signal was also detected in the adipose tissue (Fig. 5 b). No signal was detected in the samples containing organized granulomas.

For PRV-3, one heart showed positive ISH signal. In this sample, mild myocarditis in the stratum compactum and stratum spongiosum were observed. The signal was only detected in the stratum spongiosum (Fig 5 c). Although having a lower Cq value, no pathological lesions were detected in other PRV-3 positive heart samples, and no signal was detected in ISH.

In all SAV-positive skeletal muscle samples selected for ISH analyses, signal was detected in relation to intact myocytes (Fig. 5 d) and adipose tissue. No signal was detected in lesioned areas of the samples.

4. Discussion

In this study, we investigated the prevalence and the macro- and microscopical characteristics of red and melanized focal changes in rainbow trout marine farms. The prevalence of RFC and MFC in the fillets at the time of slaughter ranged from 1.46% to 6.47% in the three farms investigated. This is considerably lower than the prevalence of 5% for RFC and 20-30% for MFC that previously have been reported in farmed Atlantic salmon (Bjørgen et al., 2019; Mørkøre et al., 2015). The histological analysis showed that the dominating tissue response in farmed rainbow trout was fibrosis and muscle cell regeneration, which could be due to a different response to initial tissue damage, or a difference in the inflammatory response towards infectious agents. Furthermore, despite similarities in environmental and production-related factors between farmed Atlantic salmon and rainbow trout, these factors cannot be ruled out as potential contributors to the observed differences in MFC between the two species.

The macroscopic scoring revealed RFC and MFC of predominantly low grades and primarily located in the cranio-ventral region of the abdominal wall, aligning with findings in Atlantic salmon reported by Bjørgen et al. (2019). The shared anatomical localization suggests that the initial cause of the condition may be similar for both species. Additionally, the production procedures in both species are rather equivalent, but there are some species-specific differences related to physiology, such as rainbow trout's requirement for higher water temperature at hatching compared to salmon (Noble et al., 2020). Additionally, rainbow trout are highly active, resulting in increased energy and oxygen demand (personal communication, DVM. Magnus Lian, fish health manager, Lerøy Sjøtroll). Thus, the low prevalence of RFC and MFC in rainbow trout may be due to variations in external factors during production or an altered response to inflammation, as well as the possible presence of

infectious agents. The dominating cranio-ventral localization of RFC and MFC in Atlantic salmon has prompted speculations regarding potential local tissue-related factors that may contribute to the initial tissue damage. These factors include intraperitoneal vaccination (Koppang, Haugarvoll, Hordvik, Aune, & Poppe, 2005), costal fractures (Brimsholm et al., 2023a), and internal trauma resulting from bloating due to undigested pellets in the stomach. However, so far, no single factor has been singled out as a primary cause of these changes, and the condition is regarded as multi-factorial.

We found that several of the histological characteristics in rainbow trout were consistent with those previously reported in Atlantic salmon. These include inflammatory and repair responses, such as inflammatory cells, including melano-macrophages, and necrosis, tissue fibrosis, and myocyte regeneration. However, certain differences were notable, which rendered the classification system developed for RFC/MFC in Atlantic salmon (Bjørgen et al., 2019) somewhat challenging to apply to rainbow trout. Specifically, we rarely observed the most common categories described in salmon, such as the presence of melano-macrophages dispersed among seemingly non-affected myocytes (Category 2) or areas dominated by infiltrating inflammatory cells in fibrotic tissue (Category 5-7 and 9). Furthermore, organized granulomas (Category 8) were observed in only one fish. Instead, the predominant finding in rainbow trout appeared to be fibrosis and relatively few melano-macrophages (Category 3 and 4) and an abundant presence of small, putatively regenerative myocytes within loose connective tissue. The low abundance of melano-macrophages with advanced fibrosis and muscle regeneration can be regarded as key characteristics of MFC in rainbow trout, which is a different tissue response to that of Atlantic salmon.

The altered inflammatory response observed in rainbow trout may be attributed to specific species reactions to pathogens, and several factors, including water temperature, fish species, and the persistence of harmful agents could influence these differences. Indeed, the

effect of water temperature on regeneration is an important aspect for poikilothermic animals (Schmidt, Andersen, Ersbøll, & Nielsen, 2016). Additionally, immune cell function has been shown to be influenced by water temperature in rainbow trout (Gräns, Rosengren, Niklasson, & Axelsson, 2012), which could imply that low-temperature water could decrease the rate of an immune response, as rainbow trout has a higher preferred temperature than Atlantic salmon. Water temperature has also been suggested to influence the prevalence of MFC at different sampling points in farmed Atlantic salmon in Norway (Brimsholm et al., 2023b). We detected granulomas in one rainbow trout, which contrasts the distinct presence of organized granulomas encircled by elongated melano-macrophages which is a hallmark of severe MFC in Atlantic salmon. In Atlantic salmon, the melanin-containing M2 polarized macrophages found within the chronic inflammatory changes are associated with PRV-1 infection (Malik et al., 2021a). Additionally, organized granulomas in Atlantic salmon have been shown to contain replicating PRV-1 (Bjørgen et al. 2020), acting as a constant trigger of inflammation which can cause granuloma formation (Bjørgen et al., 2015; Malik et al., 2021a).

In Atlantic salmon, infection with PRV-1 is associated with heart and skeletal muscle inflammation (HSMI) (Wessel et al., 2017), a condition that most commonly occurs during the seawater production phase. PRV-1 infects erythrocytes during the acute stage of infection (Finstad et al., 2014), and cardiomyocytes are also affected, causing HSMI lesions primarily in the heart. Severe cases may cause necrotic myocytes and inflammation in red skeletal muscle (Kongtorp, Kjerstad, Taksdal, Guttvik, & Falk, 2004). Subsequently, macrophages remain persistently infected by the virus, as they appear unable to eliminate it (Malik et al., 2019). In contrast, in rainbow trout, PRV-1 infection in freshwater smolts only causes mild heart lesions, has a low viral replication, and is later cleared from the tissue (Purcell et al., 2020). Although we detected PRV-1 by RT-qPCR in several rainbow trout skeletal muscle

317 samples, it could be detected by ISH in skeletal muscle only in the selected sample having the
318 lowest Cq value, i.e., containing most viral RNA. Here, the virus was localized in proximity
319 to intact myocytes and adipose tissue, with no virus detected in the lesioned areas. In
320 summary, our results suggest that the reduced prevalence of MFC in rainbow trout could be
321 related to the lower prevalence of PRV-1 infection and the lower viral load in PRV-1 infected
322 individuals in rainbow trout compared to Atlantic salmon. Bjørgen et al. (2019) found that
323 low-grade RFC and MFC were preceding the detection of PRV-1, but that PRV-1 coincided
324 with increased severity in macroscopic and histological grading in MFC. The prevalence of
325 low-grade MFC in rainbow trout at slaughter, combined with the dominant presence of
326 fibrosis and skeletal muscle regeneration, aligns well with the observed differences in the
327 response to PRV-1 infection between Atlantic salmon and rainbow trout.

328 An alternative explanation for the differing prevalence of RFC and MFC in rainbow
329 trout and Atlantic salmon may be rooted in innate differences in their immune and healing
330 responses to initial hemorrhage and tissue damage, and thus not related to PRV-1 infection.
331 As of our knowledge, no comparative study on skeletal muscle has been conducted between
332 the two species. However, a study did compare lesions in the pancreas and pyloric caeca in
333 Atlantic salmon and rainbow trout following intraperitoneal injections with oil-based
334 adjuvants that did not contain antigens (Mutoloki, Reite, Brudeseth, Tverdal, & Evensen,
335 2006). In this study, the local inflammatory reaction in Atlantic salmon persisted for twice as
336 long as in rainbow trout. Furthermore, another study investigated skeletal muscle
337 regeneration during experimental trials focusing on bacterial infection in Atlantic salmon and
338 mechanical trauma in rainbow trout. The results indicated inflammatory responses and
339 upregulation of inflammatory mediators in both species, with the most robust response seen
340 in Atlantic salmon (Ingerslev, Lunder, & Nielsen, 2010). However, due to differing harmful
341 factors to which each species was exposed, direct comparative conclusions could not be

made. Regarding skeletal muscle, studies on wound healing in the dorsal muscle trunk of rainbow trout have revealed slow regeneration and an abundance of fibrosis (Schmidt et al., 2016), revealing that this species, in general, has a slow regenerative response in skeletal muscle. The elaborate fibrosis previously reported is in line with our results. In summary, several studies indicate differences in the healing responses between these two species, although the precise underlying mechanisms involved have yet to be investigated.

We conducted ISH not only for the detection of PRV-1 but also for SAV. Our results showed similar staining patterns for both viruses. Although SAV was detected in all selected samples investigated using ISH, the positive signals were not associated with the observed lesions. Instead, they were predominantly seen in proximity to intact myocytes and occasionally within adipose tissue. In Norwegian salmonid aquaculture, the subtypes SAV2 and SAV3 are the causative agents of the disease known as Pancreas disease (PD) in Atlantic salmon and rainbow trout, affecting both the pancreas and heart- and skeletal muscle (Hodneland, Bratland, Christie, Endresen, & Nylund, 2005; McLoughlin & Graham, 2007). The current PD pandemic in Norway is geographically divided based on the presence of SAV subtypes; the Northern part of the coast is free from SAV infection, SAV2 is present in the Northern part of the infected zone, while SAV3 is the causative agent in the Southern part (Sindre, Patel, Olsen & Løkslett, 2023), and there is a small overlapping region where both subtypes can be found. The three fish farms in our study were located in the Southern part where SAV3 is found. The skeletal muscle lesions observed in rainbow trout with the subtype SAV2 have been shown to have a slow regenerative capacity, likely due to the virus' tropism for skeletal muscle satellite cells (Biacchesi et al., 2016). The localization of SAV that we observed in ISH, combined with the necrotic myocytes in SAV-infected control samples, and the abundant presence of small regenerative myocytes in lesions indicate impairment of the infected satellite cells.

The impact of PRV-3 infection in our samples is believed to be limited, given that the virus was only detected in heart samples using RT-qPCR and by ISH, only in the heart with mild lesions. PRV-3 is closely related to PRV-1 and has been shown to induce cross-protection against PRV-1 infection in Atlantic salmon (Malik et al., 2021c). In rainbow trout, PRV-3 can be the causal agent of a condition with many similarities to HSMI in Atlantic salmon (Vedramin et al., 2019; Dhamotharan et al., 2018; Olsen et al., 2015), although some important differences have been reported. HSMI in rainbow trout primarily occurs during the freshwater stage of production; the infection can cause anemia (Olsen et al., 2015), and the virus is cleared more efficiently than PRV-1 in Atlantic salmon (Hauge et al., 2017; Vendramin et al., 2019). The detection of PRV-3 in our heart samples aligns with earlier reports regarding the distribution of PRV3. As PRV-3 was not detected by RT-qPCR in skeletal muscle in our study, it is possible that the virus had been cleared from the tissue.

In conclusion, our study provides insight into the prevalence and key histopathological characteristics of RFC and MFC in rainbow trout reared in a marine environment. The observation that most of these changes appear in the cranio-ventral area is consistent between rainbow trout and Atlantic salmon, suggesting a shared underlying cause. However, the lower prevalence in rainbow trout suggests differences in the impact of the causative factor(s). Notably, fibrosis and regeneration were more prominent histopathological features in MFC in rainbow trout as compared to Atlantic salmon. The co-localization of PRV-1 and melano-macrophages in Atlantic salmon MFCs and the lack of this co-localization in rainbow trout MFC may explain the lower prevalence and severity of MFC in rainbow trout. This suggests that rainbow trout may respond to the PRV-1 infection in skeletal muscle in a way that typically prevents the development of chronic inflammation leading to a reduction in both the prevalence and severity of MFC.

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Tables

Table 1. Sampling information. Abbreviations: prev. – prevalence. he. – heart, sp. – spleen, and mu. – muscle. N = number of samples.

	Farm 1 (*Laksevika*)		Farm 2 (*Tepstad*)		Farm 3 (*Djupestallen*)	
Slaughter (date)	11th of April		18th of April		2nd of May	
Melanin prev.	1.46% (9 of 616 filets)		6.47% (27 of 417 filets)		5.76% (15 of 260 filets)	
RNAlater he./sp.	N=35/29		N=26/22		N=30/30	
Formalin he./sp.	N=15/12		N=15/10		N=10/10	
	Affected fish		Affected fish		Affected fish	
	Melanized	Control	Melanized	Control	Melanized	Control
RNAlater mu.	N=9	N=5	N=13	N=10	N=8	N=8
Formalin mu.	N=9	N=5	N=10	N=10	N=10	N=10

Table 2. Target and control probes for *in situ* hybridization**.** n.c. – negative control. b.p. – base pairs

	Probe	Accession no.	Target region (b.p.)	Catalog no.
target	PRV 1	KY429945.1	415 – 1379 (L3 segment)	537451
	PRV 3	MG253809.1	81 – 1933 (L3 segment)	555221
	SAV	AY604235.1:7788-11747	1366 - 2257	1219771-C1
n.c.	DapB	EF191515	414 - 862	310043

Table 3. Prevalence of melanin according to anatomical location (abd. 1 – cranio-ventral region, abd. 2 – caudoventral region and dors. – dorsal region) and severity (from 1-8) in Farm 1–3.

	Abd. 1			Abd. 2			Dors.		
Score	Farm 1	Farm 2	Farm 3	Farm 1	Farm 2	Farm 3	Farm 1	Farm 2	Farm 3
1	3	7	5	2	4	1	0	4	3
2	1	3	0	2	0	1	0	4	2
4	1	1	0	0	0	0	0	3	2
8	0	0	1	0	0	0	0	1	0

Table 4. RT-qPCR analyzes in skeletal muscle and heart, showing mean Cq values (standard deviation).

	Farm 1	Farm 2	Farm 3
PRV-1	Mu: (n= 1/25) 34,7 He: (n= 2/16) 32,57 (± 2.26)	Mu: (n= 0/54) He: (0/10)	Mu: (n= 26/37) 33.39 (± 2.96) He: (n= 8/10) 27,02 (± 4.85)
PRV-3	Mu: (n= 0/25) He: (n= 3/16) 31.41 (± 4.41)	Mu: (n= 0/54) He: (n= 6/10) 30, 94 (± 3.90)	Mu: (n= 0/37) He: (n= 4/10) 32,70 (± 2.81)
SAV	Mu: (n= 0/25) He: (n= 0/16)	Mu: (n= 52/54) 26,88 (± 3.62) He: (n= 6/10) 32,09 (± 3.06)	Mu: (n= 9/37) 34,83 (± 1.16) He: (n= 0/10)

551 **Table 5.** *In situ* hybridization (ISH) and RT-qPCR results in tissues selected for ISH analyses.
552 Skeletal muscle (Mu) and heart (He) samples. Negative (Neg) results imply no detected signal in ISH
553 sections, positive (Pos) results imply detected signal in sample. *Muscle samples containing
554 organized granulomas.

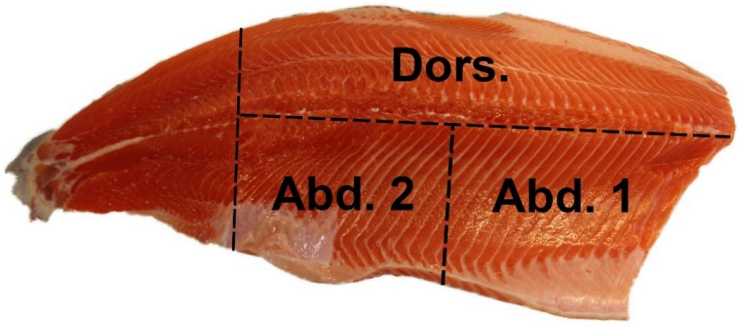
PRV-1		PRV-3		SAV	
ISH (Tissue)	Cq value	ISH (Tissue)	Cq value	ISH (Tissue)	Cq value
Neg (Mu)	34.2	Pos (He)	28.4	Pos (Mu)	20.6
Neg (Mu)	32.7	Neg (He)	24.9	Pos (Mu)	21.8
Neg (Mu)*	37			Pos (Mu)	22.5
Neg (Mu)*	37			Pos (Mu)	22.0
Pos (Mu)	26.5			Pos (Mu)	22.6

555

556

Figures

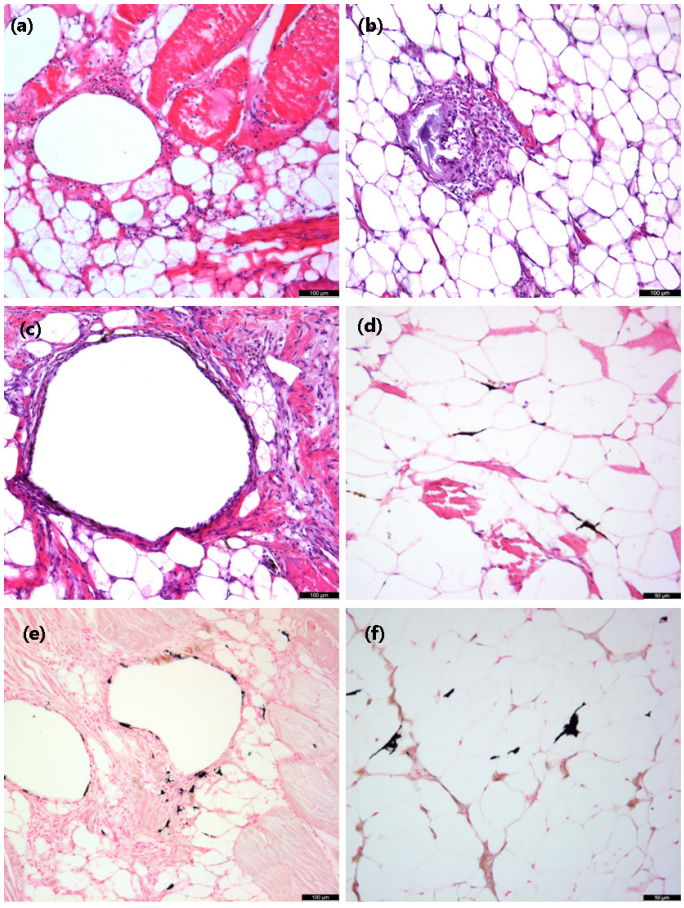
Figure 1. Regions used for registration of hemorrhagic and melanized muscle changes in farmed Rainbow trout. Registering of melanin according to anatomical location. Anatomical location: abd.1 – abdominal cranio-ventral region, abd.2 – abdominal caudoventral region and dors. - dorsal region.



563 **Figure 2.** Macroscopic grading of RFC ad MFC in rainbow trout. (a) Grade 0: No macroscopic
564 changes observed in the skeletal muscle. (b) Grade 1: Hazy changes, all sizes. (c) Grade 2: Local and
565 distinct change, <3 cm size. (d) Grade 4 RFC: Local and distinct change with red color, 3-6 cm. (e)
566 Grade 4 MFC: Local and distinct change, 3-6 cm. (f) Grade 8: Large change, >6 cm.

567

568 **Figure 3.** Histological investigations of red and melanized focal changes (RFC ad MFC). (a) RFC:
569 Hemorrhage and necrotic myocytes in white skeletal muscle. Adipocytes and vacuoles are present
570 within the lesion. HE stain, scale bar = 100 µm. (b)MFC: Organized granuloma located in the adipose
571 tissue adjacent to the skeletal muscle. Scarce number of melano-macrophages in the tissue. HE stain,
572 scale bar = 100 µm. (c) MFC: Large vacuole surrounded by elongated melano-macrophages and
573 inflammatory cells located in the white skeletal muscle. HE stain, scale bar = 100 µm. (d) MFC:
574 Melano-macrophages located between adipocytes in the myosepta. HE stain, scale bar = 50 µm. (e)
575 Positive staining for melanin in melano-macrophages surrounding a large vacuole. Fontana Masson
576 stain, scale bar= 100 µm. (f) Positive staining for melanin in melano-macrophages located in adipose
577 tissue. Fontana masson stain, scale bar = 50 µm.



578

579 **Figure 4.** Collagen stains of melanized focal changes. (a) Hematoxylin and eosin stain: Area with
580 small, regenerative myocytes interspersed in connective tissue. (b) Van Gieson's stain: Collagen-
581 stained pink, skeletal muscle stained yellow/orange, nucleus stained brown/black. (c) Masson's
582 trichrome stain: Collagen stained blue, skeletal muscle stained red, nucleus stained brown/black.

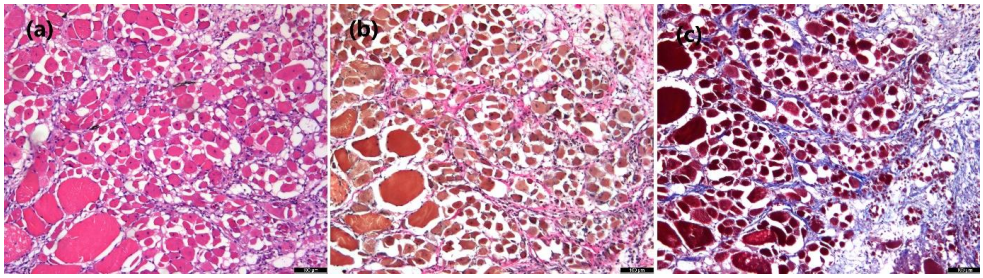
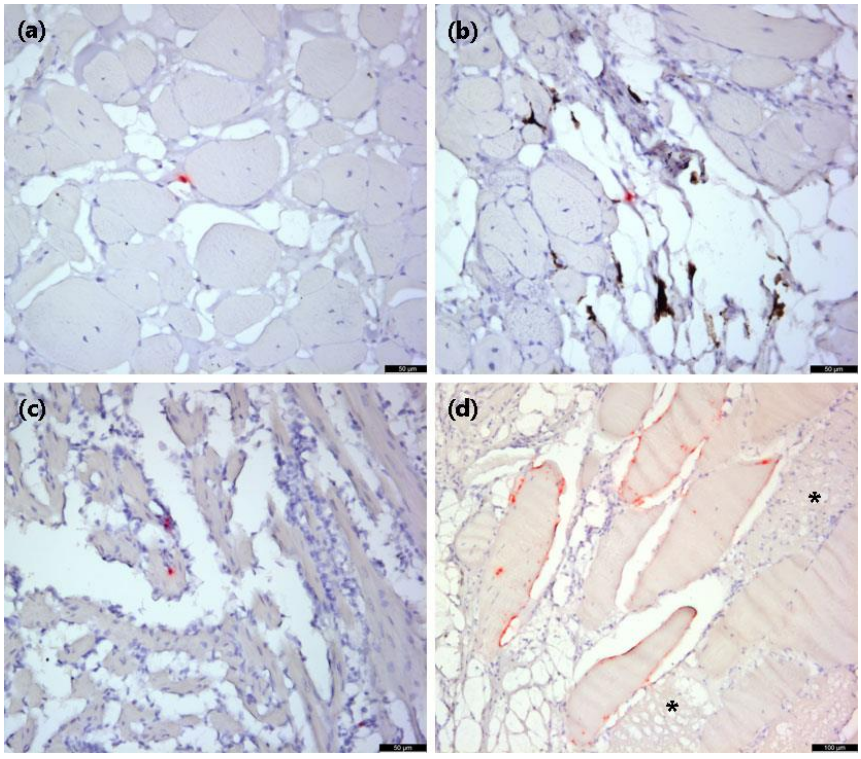
583 

Figure 5. *In situ* hybridization targeting Piscine orthoreovirus-1 (a,b), Piscine orthoreovirus-3 (c), and Salmonid alphavirus (d-e). (a) Skeletal muscle with PRV-1 positive cell located adjacent to an intact myocyte. Scale bar = 50 µm. (b) PRV-1 signal detected in the adipose tissue adjacent to intact muscle fibers. Note that no signal is detected within melano-macrophages. Scale bar =50 µm. (c) PRV-1 signal in cells in the *stratum spongiosum* of the heart. Scale bar = 50 µm. (d) Skeletal muscle with SAV-positive reactivity. No signal detected in lesioned areas or necrotic myocytes (asterisk). Scale bar = 100 µm.



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