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

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ABSTRACT: Fillet discoloration by red and melanized focal changes (RFCs and MFCs) 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 encompassing 1293 rainbow trout from 3 different farm sites in Norway, all examined at the time of slaughter. Both macroscopic and histological assessments of the changes were performed. Reverse transcription (RT)-qPCR analyses and *in situ* hybridization (ISH) were used to detect the presence and location, respectively, of potential viruses. Only 1 RFC was detected in a single fillet, while the prevalence of MFCs ranged from 1.46 to 6.47% between populations. 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 1 fish. Notably, the presence of inflammatory cells, including melano-macrophages, appeared lower compared to what has been previously documented in Atlantic salmon MFCs. 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, rainbow trout develop MFCs of a different character than farmed Atlantic salmon, and we speculate whether the observed pathological differences are contributing to their reduced occurrence in farmed rainbow trout.

KEY WORDS: Fillet · Melanin · Melano-macrophage · Rainbow trout · Skeletal muscle · Piscine orthoreovirus 1 · Salmonid alphavirus

1. INTRODUCTION

With an approximate prevalence of 20% in the Norwegian Atlantic salmon *Salmo salar* production, the occurrence of melanized focal changes (MFCs) 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 et al. 1997). Melano-macrophages can be found in lymphoid organs and inflamed tissues of fish (Agius 1981, Thorsen et al. 2006, Larsen et al. 2012). While severe melanized white muscle changes in salmon have been associated with piscine orthoreovirus 1 (PRV-1) infection, they can also occur independ-

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ently of virus presence (Bjørgen et al. 2015, 2019). The MFCs can be preceded by focal hemorrhagic lesions (red focal changes, RFCs) that may progress into chronic inflammatory changes containing melano-macrophages over time (Bjørgen et al. 2019). MFCs can occur as only sparse discoloration or as heavily pigmented lesions. 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. 2015, 2019). The initiating causes of the RFCs remain undetermined (Bjørgen et al. 2019).

MFCs have also been noted in 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 MFCs 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 susceptibilities of rainbow trout and Atlantic salmon to viral infections differs, which may be of importance for viruses inducing inflammation in skeletal muscle and being prevalent in aquaculture of salmonids in Norway, i.e. salmonid alphavirus (SAV) and PRV. PRV-1 to -3 and SAV1 to -6 subtypes infect many salmonid species and appear to cause species-specific diseases dependent on virus subtype. PRV and SAV can be found in the same individual fish (Wiik-Nielsen et al. 2016). PRV-1 infection is endemic in seawater-farmed Atlantic salmon and may lead to heart- and skeletal-muscle inflammation (HSMI), one of the most common viral diseases in Norwegian aquaculture (Olsen & Dahle 2023). PRV-2 causes erythrocytic inclusion body syndrome in coho salmon *O. kisutch* in Japan (Takano et al. 2024), while PRV-3 causes heart inflammation in rainbow trout (Vendramin et al. 2019) and has occasionally caused outbreaks in rainbow trout freshwater facilities, characterized by both heart inflammation and anemia (Olsen et al. 2015, Adamek et al. 2019), and has been associated with jaundice syndrome in farmed coho salmon in Chile (Solarte-Murillo et al. 2023). The relative high prevalence of PRV-3 in brown trout *Salmo trutta* and the findings of PRV-3 in brown trout in a wide geographic area indicates that brown trout may be a natural reservoir of PRV-3 (Garseth et al. 2013, Pojezdal et al. 2020). In contrast, PRV-1 variants found on the west coast of the North American continent have not been associated with disease in farmed rainbow trout (Purcell et al. 2020), and PRV-3 does not lead to disease in Atlantic salmon after experimental infection (Malik et al. 2021b). Whereas Atlantic salmon remain persistently infected by PRV-1, rainbow trout appear to be able to fight off PRV-1 and -3 after a period of infection (Hauge et al. 2017). Several 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 et al. 2023).

In the present study, we investigated focal melanized changes in 3 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 MFCs 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 MFCs 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 (60.5302° N, 5.1158° E), Farm 2 in Tepstad (60.5549° N, 5.3818° E), and Farm 3 in Djupestallen (60.0590° N, 5.4527° E). Fish were slaughtered during the spring of 2017 and were provided by Lerøy Vest AS/Sjøtroll Havbruk AS and Blom Fiskeoppdrett. Fish from Farm 1 were investigated on 11 April and consisted of 616 individuals sorted in the weight category 4–5 kg with a total of 9 muscle changes. Farm 2 was investigated on 18 April and included of 417 individuals in the weight category 5–6 kg with a total of 27 muscle changes. Farm 3 was investigated on 2 May and consisted of 260 individuals in the weight cate-

gory 4–5 kg with a total of 15 muscle changes. At slaughter, muscle abnormalities were registered according to anatomical location (Fig. 1), and the severity of observed macroscopic changes (on a scale of 1–8 according to Mørkøre 2012) was noted. Fillets with no changes received a score of 0. Fillets exhibiting diffuse discolorations of all sizes were assigned a score of 1. Demarcated spots smaller than 3 cm were scored 2, while those between 3 and 6 cm were scored 4. Larger melanized areas, ⩾6 cm, were assigned a score of 8.

2.2. Sampling for reverse transcription (RT)-qPCR and histological analysis

To test the populations for the presence of PRV-1, PRV-3, and SAV, hearts were collected and stored in RNAlater at the slaughter line (Table 1). To test RFCs/MFCs for virus presence, affected muscle tissue was collected and stored in RNAlater. Control tissues, 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 in 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 (https://www.patogen.no/nb/), 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. Samples were defined as positive when having a threshold cycle (Ct) value below 37.0.

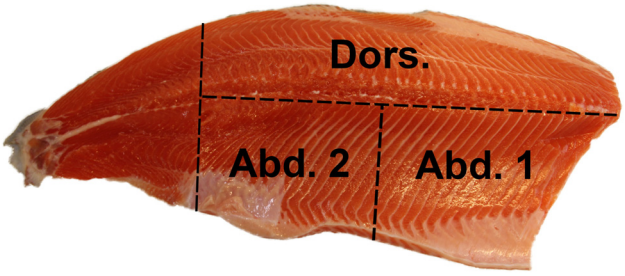

Fig. 1. Regions used for registration of hemorrhagic and melanized muscle changes in farmed rainbow trout. Melanin was registered according to anatomical location (Abd. 1: abdominal cranio-ventral region; Abd. 2: abdominal caudo-ventral region; Dors.: dorsal region)

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 (Table 1) 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. Based on results from the HE-stained sections, selected samples (n = 10) were stained using Van Gieson stain and Masson trichrome stain for the detection of collagen, and Fontana Masson stain for the detection of melanin.

2.5. *In situ* hybridization (ISH)

RNAscope 2.5 HD Assay-red (Advanced Cell Diagnostics) was used for *in situ* hybridization (ISH) following the manufacturer's guidelines (Wang et al. 2012). In brief, 4 µm thick paraffin-embedded

Table 1. Rainbow trout samples obtained from the 3 different farm sites. Both fillets with melanized changes and fillets devoid of changes, i.e. controls, were obtained. n = number of samples

Farm (location)	Slaughter date (2017)	Melanin prev. (%)	Heart samples (n)		Muscle samples (n)		
			RNAlater	Formalin	Status	RNAlater	Formalin
Farm 1 (Laksevika)	11 April	1.46 (9/616 fillets)	35	15	Melanized	9	9
					Control	5	5
Farm 2 (Tepstad)	18 April	6.47 (27/417 fillets)	26	15	Melanized	13	10
					Control	10	10
Farm 3 (Djupestallen)	2 May	5.76 (15/260 fillets)	30	10	Melanized	8	10
					Control	8	10

tissue sections were prepared from 11 fish. The samples were selected based on their histological characteristics as evaluated in HE staining, combined with detectable virus levels of PRV-1, PRV-3, or SAV (quantification cycle [Cq] values of <37) in the tissue. Due to high Cq values and low prevalence of lesions, no samples from Farm 1 were selected for ISH analyses. From Farm 2, we selected 3 skeletal muscle samples with a Cq value ⩽34.2 for PRV-1. Two samples with organized granulomas from one fish were added, although these samples had a Cq value ⩾37 for all investigated viruses. Two heart samples were selected from Farm 2, one with a Cq value of 28.4 for PRV-3 and mild cardiac lesions, and one with a Cq value of 24.92 for PRV-3 and 30.85 for SAV, with no pathological lesions detected in histological examination. As Farm 3 represented the lowest Cq values for SAV in skeletal muscle, 5 skeletal muscle samples were selected for ISH detection of SAV in the tissue.

The slides were mounted on positively charged glass slides (Superfrost©; Mentzel) and air-dried for a minimum of 24 h before being subjected to a 60°C incubation for 90 min, followed by 2 rounds of 5 min treatment with xylene and 2 rounds of 1 min treatment with 100% ethanol for dewaxing. Subsequently, the samples underwent a 10 m treatment with hydrogen peroxide to block endogenous peroxidase activity. The pretreatment included heat treatment in RNAscope® Target Retrieval Reagent at 100°C for 15 min, followed by a protease treatment for 10 min at 40°C to permeabilize the cells.

For hybridization, the slides were incubated with the target ZZ-probes (Table 2) for 2 h at 40°C. Signal amplification was obtained by sequentially incubating the slides with the 6 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). 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 3 viruses, infected tissue from Atlantic salmon was utilized (Fig. A1 in the Appendix). 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

Macroscopic grading was performed in fillets at all 3 farm sites (Fig. 2). Farm 1 had an MFC prevalence of 1.46%. One individual also displayed a focal hemorrhage, deemed as an RFC. Farms 2 and 3 had an MFC prevalence of 6.47 and 5.76%, respectively. No RFCs were observed in these 2 farms. In all farms, no transient forms between RFC and MFC were observed. MFCs were primarily detected in the cranio-ventral region and the dorsal part of the fillet, and the changes were generally of a low severity. For details, see Table 3.

The severity of the changes followed the same trend in all 3 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 2 grade 8 changes were detected (Fig. 2).

Table 2. Target and control probes for *in situ* hybridization in rainbow trout tissues. n.c.: negative control; PRV-1 and -3: piscine orthoreovirus 1 and 3; SAV: salmonid alphavirus; DapB: targets mRNA transcripts of the dihydrodipicolinate reductase gene of *Bacillus subtilis*

	Probe	Accession no.	Target region (bp)	Catalogue 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

3.2. RT-qPCR

PRV-1 was detected in 1 muscle sample from Farm 1 with a Cq value of 34.7, and in 2 heart samples, with a mean (±SD) Cq value of 32.6 ± 2.26 (Table 4). All affected muscle samples were negative for PRV-3 and SAV. In 3 heart samples, PRV-3 was detected with a mean Cq value of 31.41 ± 4.41. None of the heart samples was positive for multiple viruses.

All muscle samples from Farm 2 were negative for PRV-1 and PRV-3. For SAV, only 2 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 6 out of 10 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 4 out of 10 heart samples, with a mean Cq value of 32.7 ± 2.81. PRV-1 was detected in 8 out of 10 heart samples, with a mean Cq value of 27.0 ± 4.85. In 4 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 Tables S1–S3 in the Supplement at www.int-res.com/articles/suppl/d158p201_supp.xlsx). Control samples, RT-qPCR negative for all viruses, showed no pathological changes.

When combining the macroscopic scoring with the histology, the histological characteristics within

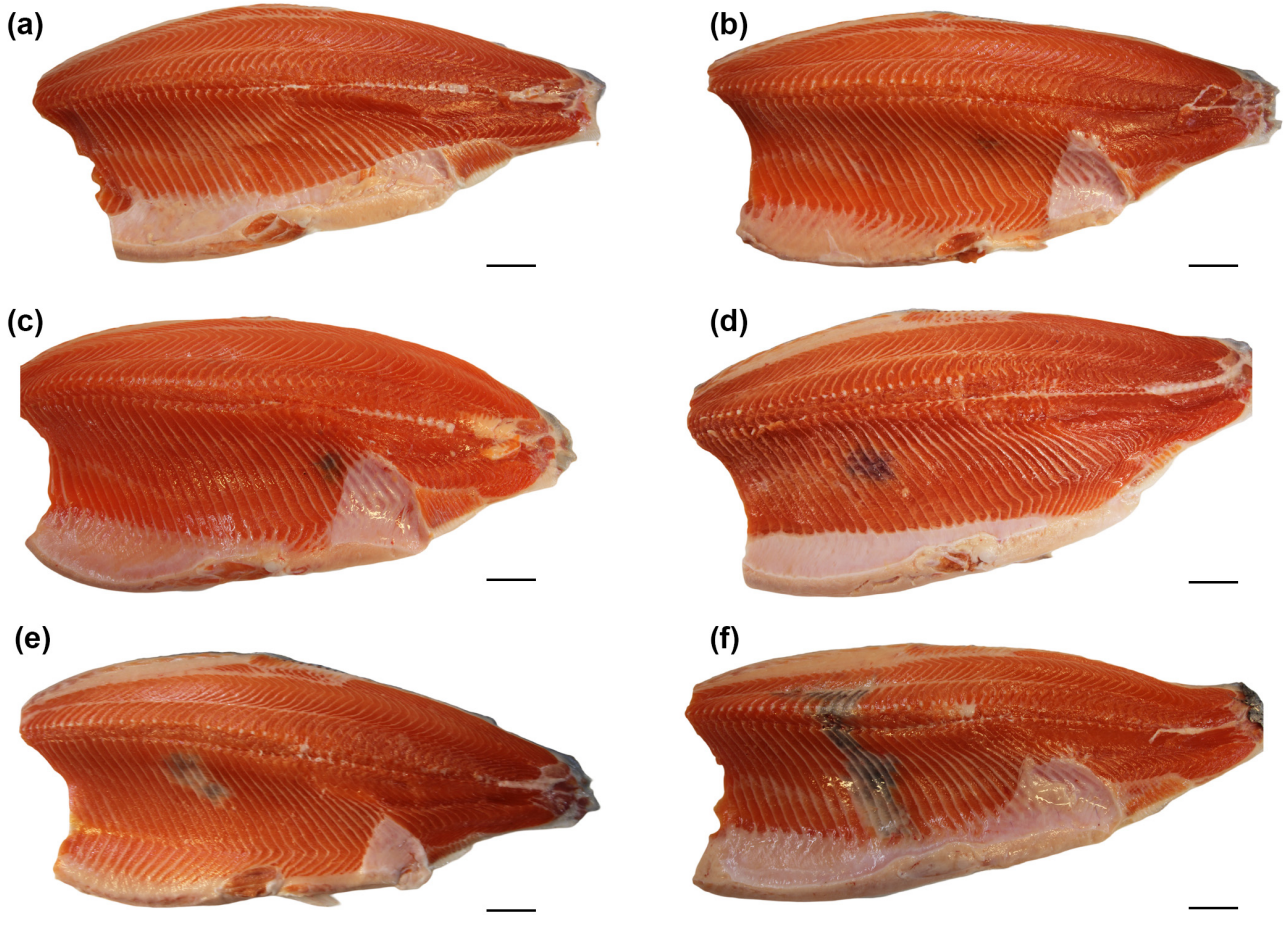

Fig. 2. Macroscopic grading of red and melanized focal changes (RFCs and MFCs) in farmed rainbow trout. (a) Grade 0: no macroscopic changes observed in the skeletal muscle. (b) Grade 1: hazy changes, all sizes. (c) Grade 2: local and distinct change, <3 cm size. (d) Grade 4 RFC: local and distinct change with red color, 3–6 cm. (e) Grade 4 MFC: local and distinct change, 3–6 cm. (f) Grade 8: large change, >6 cm. All scale bars = 4 cm

Table 3. Prevalence of melanin in rainbow trout according to anatomical location (Abd. 1: cranio-ventral region; Abd. 2: caudo-ventral region; Dors.: dorsal region, as shown in Fig. 1) and severity (scores 1, 2, 4, and 8, as shown in Fig. 2) in Farms 1–3 (see Table 1 for overall sample sizes)

Score	Abd. 1			Abd. 2			Dors.		
	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 analyses in skeletal muscle and heart of rainbow trout, showing mean Cq values ± SD. The numbers of positive samples compared to tested samples are shown in brackets. PRV-1 and -3: piscine orthoreovirus 1 and 3; SAV: salmonid alphavirus. See Table 1 for information regarding sampling of the different farms

Virus	Farm 1		Farm 2		Farm 3	
	Muscle	Heart	Muscle	Heart	Muscle	Heart
PRV 1	34.7 (n = 1/25)	32.6 ± 2.3 (n = 2/16)	(n = 0/54)	(n = 0/10)	33.4 ± 3.0 (n = 26/37)	27.0 ± 4.9 (n = 8/10)
PRV 3	(n = 0/25)	31.4 ± 4.4 (n = 3/16)	(n = 0/54)	30.9 ± 3.9 (n = 6/10)	(n = 0/37)	32.7 ± 2.8 (n = 4/10)
SAV	(n = 0/25)	(n = 0/16)	26.9 ± 3.6 (n = 52/54)	32.1 ± 3.1 (n = 6/10)	34.8 ± 1.2 (n = 9/37)	(n = 0/10)

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.

An overview of the histological organization of white skeletal muscle in rainbow trout is shown in Fig. 3. The histological changes observed in the single RFC included hemorrhage and necrosis in the skeletal muscle (Fig. 4a). In MFCs, only 1 fish contained organized granulomas (Fig. 4b), while vacuoles were often abundant in the lesioned areas (Fig. 4c). Melano-macrophages were observed surrounding the vacuoles and in the adipose tissue interspersed in the skeletal muscle and myosepta (Fig. 4d). The Fontana Masson stain confirmed the presence of melanin (Fig. 4e,f). In general, very scarce inflammatory infiltrates were observed. Instead, there was a prominent presence of loose connective tissue dispersed between

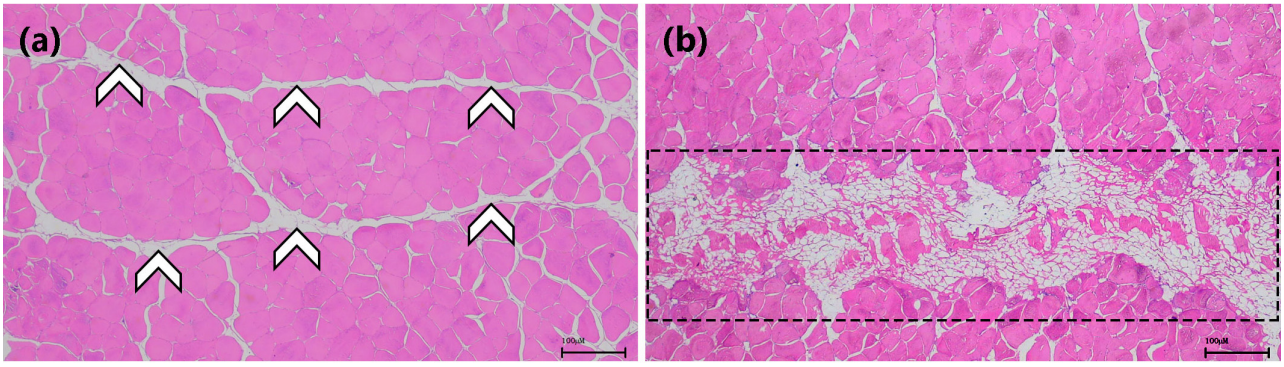

Fig. 3. Histological organization of the white skeletal muscle in farmed rainbow trout. Hematoxylin and eosin staining. (a) Myotomes appearing in cross section with multiple myocytes organized in bundles surrounded by adipose tissue, i.e. perimysium (arrowheads). (b) Myosepta appearing as a wide connective tissue structure, mainly containing abundant adipocytes (boxed). Bundles of myocytes with surrounding perimysium on each side make up the connecting myotomes. Both scale bars = 100 µm

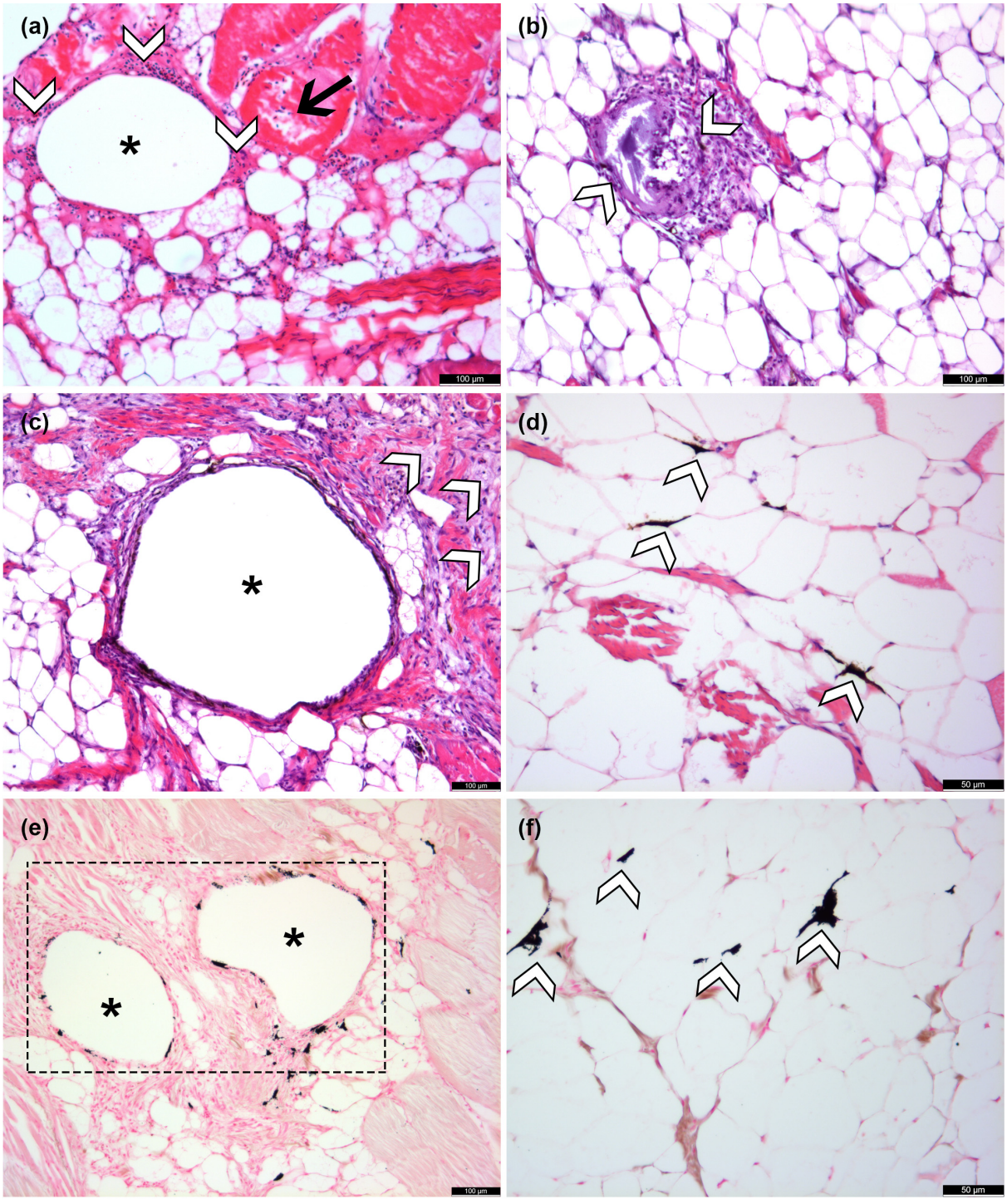

Fig. 4. Histological investigations of red and melanized focal changes (RFCs and MFCs) in farmed rainbow trout. (a) RFC: hemorrhage (arrowheads) and degenerated myocytes (arrow) in white skeletal muscle. Adipocytes and vacuoles (asterisk) are present within the lesion. HE stain. (b) MFC: organized granuloma located in the adipose tissue adjacent to the skeletal muscle. Scarce number of melano-macrophages (arrowheads) in the tissue. HE stain. (c) MFC: large vacuole (asterisk) surrounded by elongated melano-macrophages and inflammatory cells, including foam-like macrophages (arrowheads), located in the white skeletal muscle. HE stain. (d) MFC: melano-macrophages (arrowheads) located between adipocytes in the myosepta. HE stain. (e) Positive staining for melanin in melano-macrophages (within boxed region) surrounding a large vacuole (asterisk). Fontana Masson stain. (f) Positive staining for melanin in melano-macrophages (arrowheads) located in adipose tissue. Fontana Masson stain. Scale bars = (a–c,e) 100 µm, (d,f) 50 µm

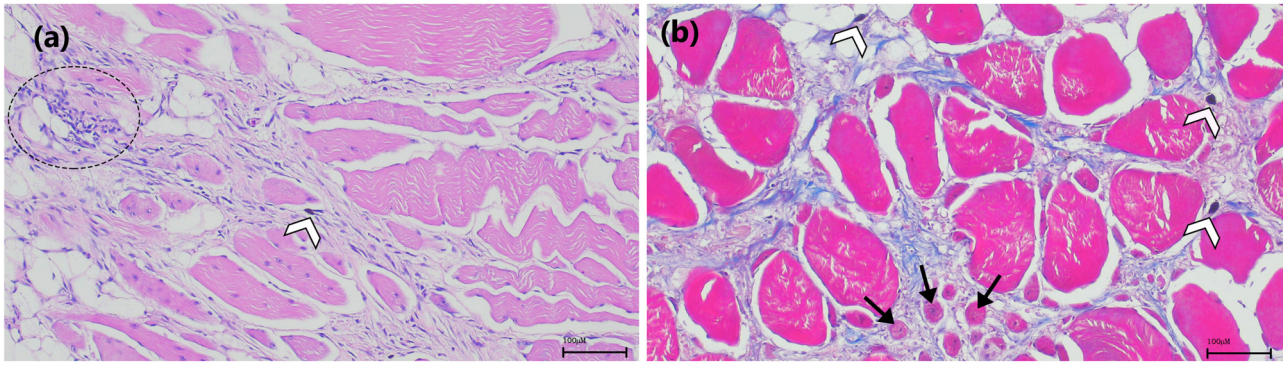

Fig. 5. Presence of collagen in melanized focal changes from farmed rainbow trout. (a) Myocytes are interspersed in a connective tissue with occasional melano-macrophages (arrowhead). An infiltrate of leukocytes is present in the dotted circle. Hematoxylin and eosin stain. (b) Collagen stained blue, skeletal muscle stained red, nucleus stained brown/black. Arrowheads indicate the presence of melano-macrophages, and arrows show the presence of small, regenerative myocytes. Masson's trichrome stain. Both scale bars = 100 µm

Table 5. Individual *in situ* hybridization (ISH) and reverse transcription-qPCR quantification cycle (Cq) results in tissues from rainbow trout selected for ISH analyses. ISH was only performed in muscle tissue for piscine orthoreovirus 1 (PRV-1) and salmonid alphavirus (SAV), and only in heart tissue (n = 2) for PRV-3. Negative (–) results imply no detected signal in ISH sections, positive (+) results imply detected signal in sample

PRV 1		PRV 3		SAV	
ISH	Cq value	ISH	Cq value	ISH	Cq value
–	34.2	+	28.4	+	20.6
–	32.7	–	24.9	+	21.8
–[a]	37			+	22.5
–[a]	37			+	22.0
+	26.5			+	22.6

[a]Muscle samples contained organized granulomas

putatively regenerated myocytes seen as small myocytes in areas adjacent to intact skeletal muscle (Fig. 5). Collagen fibers were detected by Van Gieson stain and Masson trichrome stain, with both colors revealing similar results (Fig. 5).

3.4. 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. 6a). Signal was also detected in the adipose tissue (Fig. 6b). No signal was detected in the samples containing organized granulomas.

For PRV-3, 1 heart showed a 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. 6c). 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. 6d) 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 RFCs in the fillets at the time of slaughter was negligible and the prevalence of MFCs ranged from 1.46 to 6.47%, respectively, in the 3 farms investigated. This is considerably lower than the prevalence of 5% for RFCs and 20–30% for MFCs that have previously been reported in farmed Atlantic salmon (Mørkøre et al. 2015, Bjørgen et al. 2019). 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 MFCs between the 2 species.

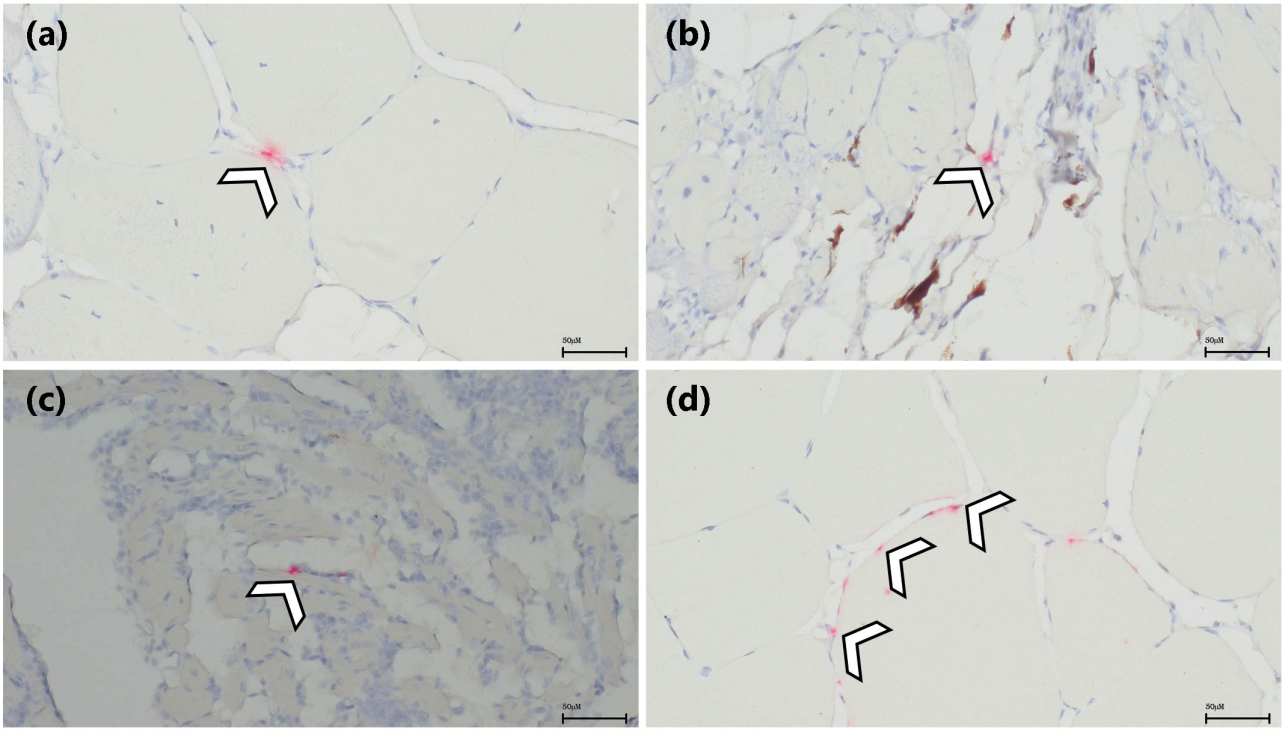

Fig. 6. *In situ* hybridization targeting (a,b) piscine orthoreovirus 1 (PRV-1), (c) PRV-3, and (d) salmonid alphavirus in rainbow trout. (a) Skeletal muscle with a PRV-1 positive cell (arrowhead) located adjacent to an intact myocyte. (b) PRV-1 signal (arrowhead) detected in the adipose tissue adjacent to intact muscle fibers. Note that no signal is detected within melano-macrophages. (c) PRV-3 signal (arrowhead) in cells in the stratum spongiosum of the heart. (d) Skeletal muscle with SAV-positive reactivity (arrowheads). No signal detected in lesioned areas or necrotic myocytes. All scale bars = 50 µm

The macroscopic scoring revealed MFCs of predominantly low grades and primarily located in the cranio-ventral region of the abdominal wall, aligning with findings in Atlantic salmon reported previously (Bjørgen et al. 2019). Only 1 RFC was detected, also in the cranio-ventral region of the fillet. 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, although there are some species-specific differences related to physiology, such as the requirement of rainbow trout 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 (M. Lian & L. Sjøtroll pers. comm.). Thus, the low prevalence of RFCs and MFCs in rainbow trout, observed in the material collected for this study but also by rainbow trout farmers in general, 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 RFCs and MFCs 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 et al. 2005), costal fractures (Brimsholm et al. 2023a), trauma to the adipose tissue and internal trauma resulting from bloating due to undigested pellets in the stomach. However, so far, no individual 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, myocyte regeneration, and changes in the adipose tissue of the myosepta. However, certain differences were notable, which rendered the classification system developed for RFCs/MFCs in Atlantic salmon (Bjørgen et al. 2019) 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 inflam-

matory cells in fibrotic tissue (Categories 5–7 and 9). Furthermore, organized granulomas (Category 8) were observed in only 1 fish. Instead, the predominant finding in rainbow trout appeared to be fibrosis and relatively few melano-macrophages (Categories 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 differs from the more heterogenic tissue response seen in Atlantic salmon.

The predominant tissue response observed in rainbow trout may be attributed to species-specific reactions to pathogens, influenced by factors such as water temperature and other environmental factors. Indeed, the effect of water temperature on regeneration is an important aspect for poikilothermic animals (Schmidt et al. 2016). Additionally, immune cell function is influenced by water temperature in rainbow trout (Gräns et al. 2012), which could imply that low-temperature water could decrease the rate of an immune response, as rainbow trout have a higher preferred temperature than Atlantic salmon. Water temperature has also been suggested to influence the prevalence of MFCs at different sampling points in farmed Atlantic salmon in Norway (Brimsholm et al. 2023b). We detected granulomas in 1 rainbow trout, which contrasts the distinct presence of organized granulomas encircled by elongated melano-macrophages, which is a hallmark of severe MFCs 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 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 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 et al. 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 samples, it could be detected by ISH in skeletal muscle only in the selected sample having the lowest Cq value, i.e. containing the most viral RNA. Here, the virus was localized in proximity to intact myocytes and adipose tissue, with no virus detected in the lesioned areas. In summary, our results suggest that the reduced prevalence of MFCs in rainbow trout could be related to the lower prevalence of PRV-1 infection and the lower viral load in PRV-1-infected individuals in rainbow trout compared to Atlantic salmon. Bjørgen et al. (2019) found that low-grade RFCs and MFCs preceded the detection of PRV-1, but that PRV-1 coincided with increased severity in macroscopic and histological grading in MFCs. The prevalence of low-grade MFCs in rainbow trout at slaughter, combined with the dominant presence of fibrosis and skeletal muscle regeneration, aligns well with the observed differences in the response to PRV-1 infection between Atlantic salmon and rainbow trout.

An alternative explanation for the differing prevalence of RFCs and MFCs in rainbow trout and Atlantic salmon may be rooted in innate differences in their immune and healing responses to initial hemorrhage and tissue damage, and thus not related to PRV-1 infection. To our knowledge, no comparative study on skeletal muscle has been conducted between the 2 species. However, Mutoloki et al. (2006) did compare lesions in the pancreas and pyloric caeca in Atlantic salmon and rainbow trout following intraperitoneal injections with oil-based adjuvants that did not contain antigens. In this study, the local inflammatory reaction in Atlantic salmon persisted for twice as long as in rainbow trout. Furthermore, another study investigated skeletal muscle regeneration during experimental trials focusing on bacterial infection in Atlantic salmon and mechanical trauma in rainbow trout. The results indicated inflammatory responses and upregulation of inflammatory mediators in both species, with the most robust response seen in Atlantic salmon (Ingerslev et al. 2010). However, due to differing harmful 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), indicating 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 2 species, although the pre-

cise 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 PD in Atlantic salmon and rainbow trout, affecting both the pancreas and heart and skeletal muscle (Hodneland et al. 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, and SAV3 is the causative agent in the southern part (Sindre et al. 2023), and there is a small overlapping region where both subtypes can be found. The 3 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 tropism of the virus 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. 2021b). In rainbow trout, PRV-3 can be the causal agent of a condition with many similarities to HSMI in Atlantic salmon (Olsen et al. 2015, Dhamotharan et al. 2018, Vendramin et al. 2019), 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 PRV-3. 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 prior to our investigations.

In conclusion, our study provides insight into the prevalence and key histopathological characteristics of MFCs 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 MFCs 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 MFCs may explain the lower prevalence and severity of MFCs in rainbow trout. This suggests that rainbow trout may respond to 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 MFCs.

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Appendix

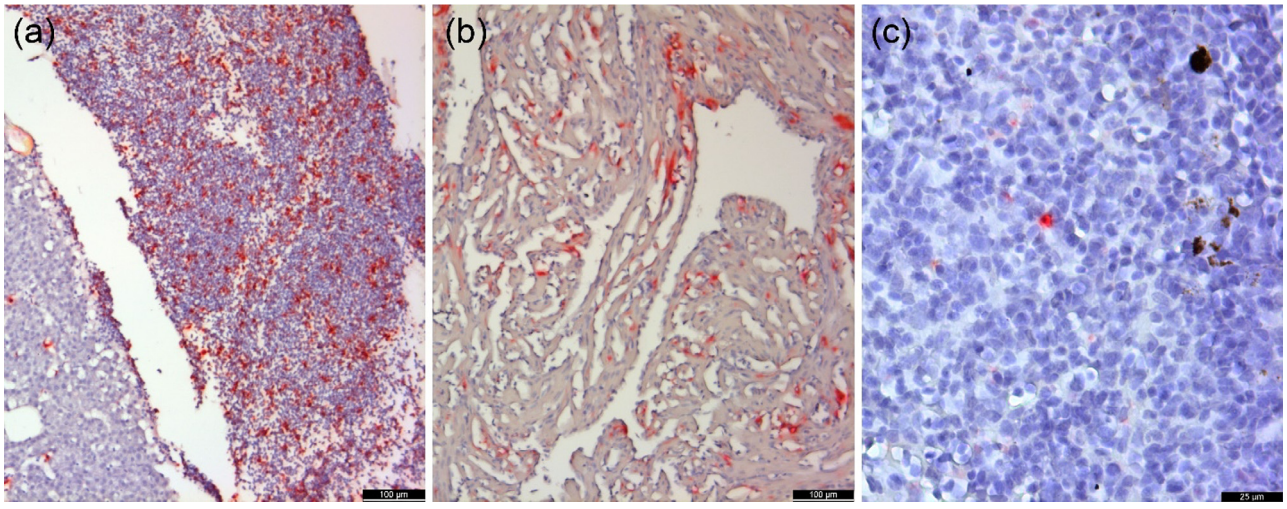

Fig. A1. Positive controls for *in situ* hybridization for PRV-1, SAV and PRV-3. (a) PRV-1 expression (red reaction) in the liver and kidney, Atlantic salmon. (b) SAV expression (red reaction) in the heart ventricle, Atlantic salmon. (c) PRV-3 expression (red reaction) in the spleen, rainbow trout. Negative control was negative (data not shown). Scale bars = (a,b) 100 µm, (c) 25 µm

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