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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.

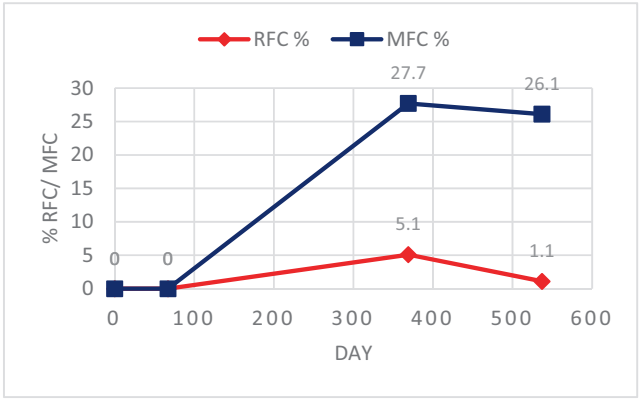

FIGURE 1 Prevalence of red (RFC) and melanized (MFC) focal changes at each sampling point (day 67, 369 and 537).

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

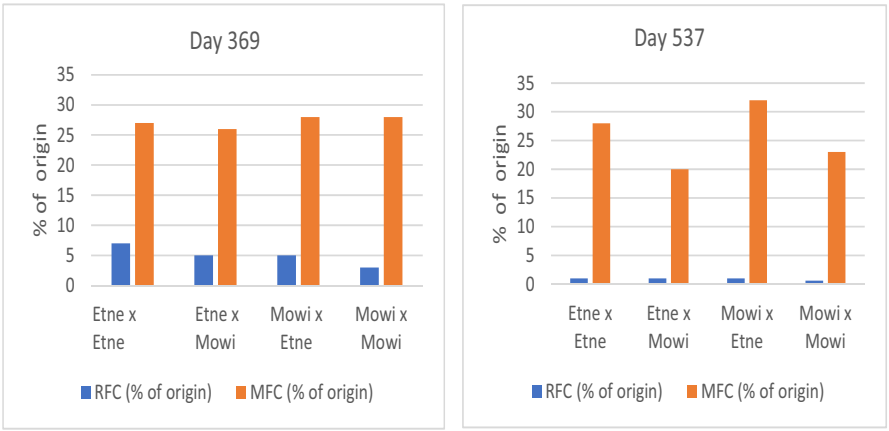

FIGURE 2 Distribution of red (RFC) and melanized (MFC) focal changes (%) between experimental groups at day 369 (a) and day 537 (b).

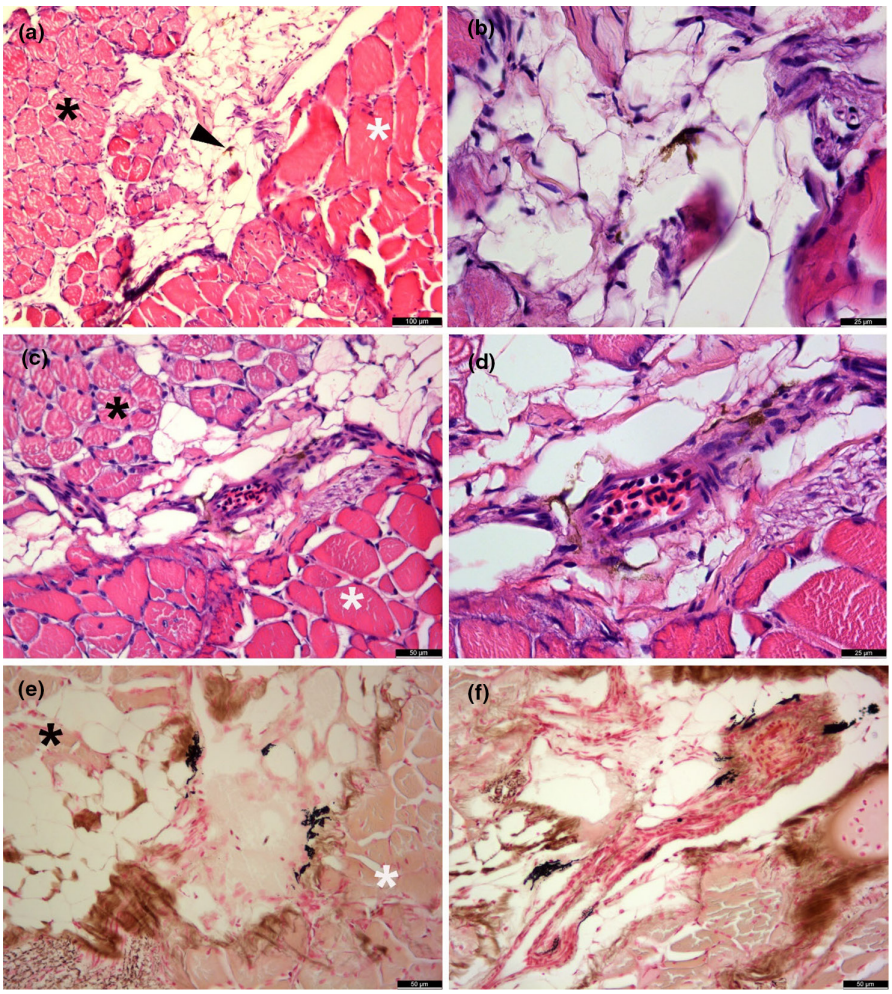

FIGURE 3 Histological changes in skeletal muscle in fish sampled at day 67. No macroscopic discoloration observed. (a) Farmed salmon (Mowi × Mowi). Area between red (black asterisk) and white (white asterisk) skeletal muscle. Melano-macrophage (arrowhead) interspersed between adipocytes. HE stain, scale 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

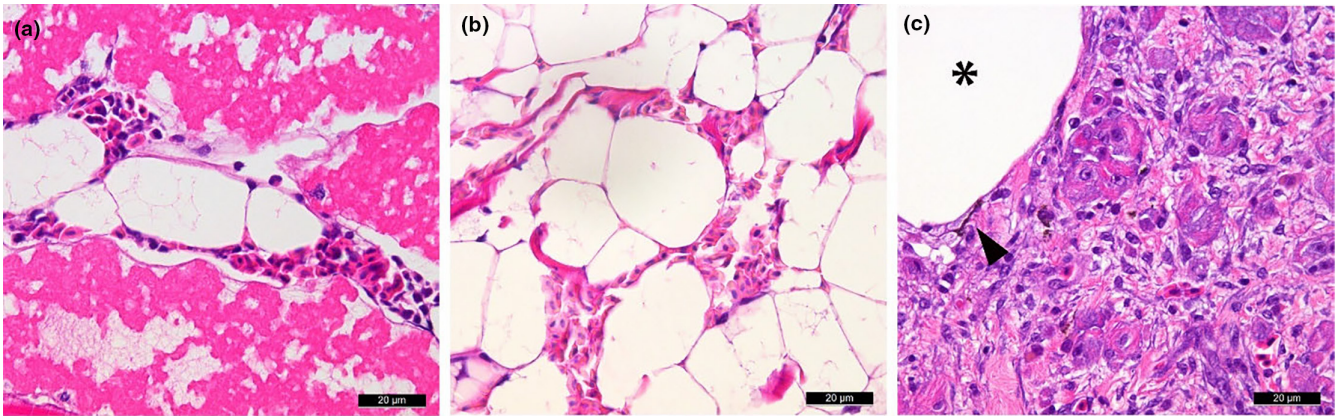

FIGURE 4 Histological changes in red focal changes (RFC).HE stain. Hybrid1 (Etne × MowiOWI), sampled at day 369 selected for illustration. (a) Erythrocytes and inflammatory cells in adipose tissue between necrotic white skeletal myocytes. Scale bar = 20 µm. (b) Area with haemorrhage in adipose tissue 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.

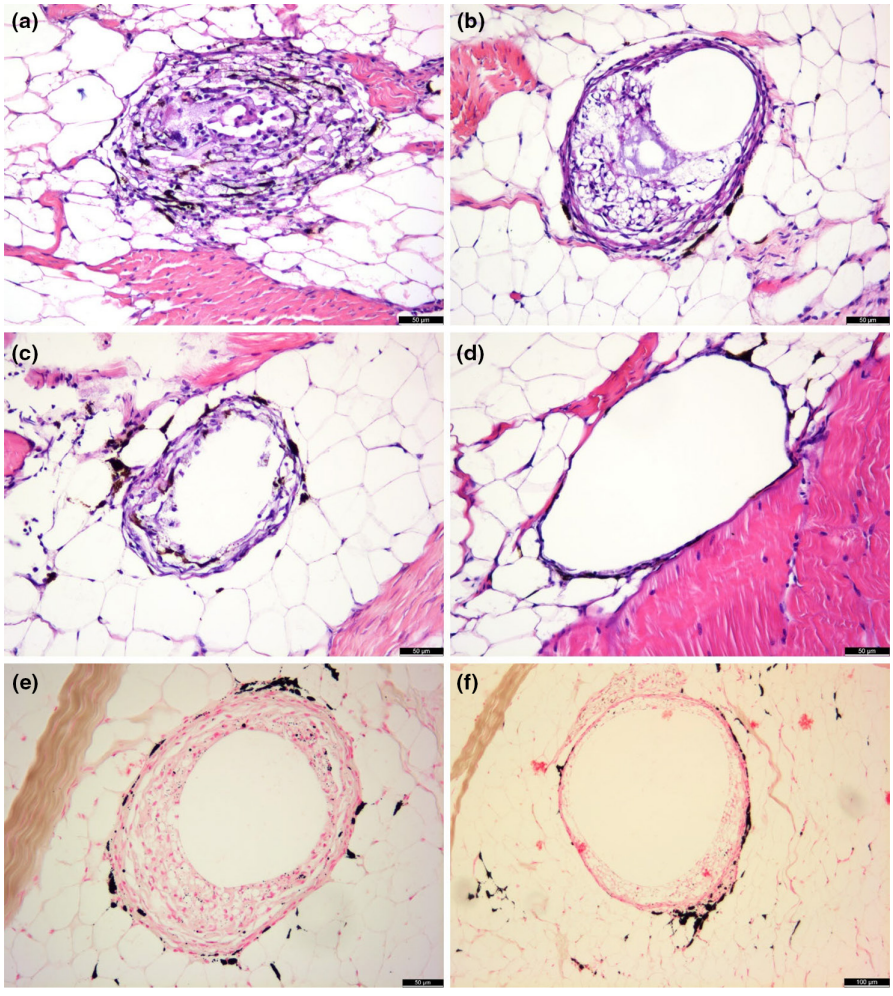

FIGURE 5 Histological changes seen in MFC at day 537. (a) Hybrid1 (Etne × Mowi). Well-organized, cell-rich granuloma, with elongated melano-macrophages and macrophage-like cells. HE stain, scale bar = 50 µm. (b) Hybrid2 (Mowi × Etne). Granuloma with central vacuole and surrounding melano-macrophages 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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