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# Red mark syndrome in rainbow trout (*Oncorhynchus mykiss*): etiological and diagnostic investigations

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# RED MARK SYNDROME IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): ETIOLOGICAL AND DIAGNOSTIC INVESTIGATIONS

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*Massimo*

# List of abbreviations

API: Italian Fish Farmers Association

BA: Blood Agar

BKD: Bactrial kidney disease

ddPCR: digital droplet polymerase chain reaction

DTU: Danish Technical University

EAFP: European Association of Fish Pathologists

eDNA: environmental DNA

FAM-labeled: Fluorescein amidites-labeled

IHC: Immunohistochemistry

IHN: Infective Hematopoietic necrosis

IPN: Infective Pancreatic Necrosis

MS-222: Tricaine methanesulfonate

MLO: Midichloria-like organism

RAS: recirculating aquaculture syndrome

RLO: rickettsia-like organism

RMS: red mark syndrome

RTFS: rainbow trout fry syndrome

SIPI: Italian Fish Pathologists Association

TEM: Transmission electron microscopy

PCNA: Proliferating Cell Nuclear Antigen

PIT: Passive Integrated Transponder

WWSD: warm water strawberry disease

VHS: Viral Hemorrhagic Septicemia

# List of papers

1. **Orioles M.,** Tomè P., Schmidt J. G., Vendramin N., Galeotti M. (2022) *A questionnaire-based survey on the presence of red mark syndrome in Italian rainbow trout (Oncorhynchus mykiss) farms.* (accepted for publication on European fish pathologist bulletin; awaiting for DOI)

2. **Orioles M**, Saccà E, Metselaar M, Bulfoni M, Cesselli D, Galeotti M. (2022) *Observations on Red Mark Syndrome in juvenile rainbow trout farmed in RAS system*. J Fish Dis. Aug 14. https://doi.org/10.1111/jfd.13707

3. **Orioles M.**, Galeotti M., Saccà E., Michela Bulfoni, Mirco Corazzin, Serena Bianchi, Diana Torge, Guido Macchiarelli, Gian Enrico Magi, Jacob Gunther Schmidt, (2022) *Effect of temperature on transfer of Midichloria-like organism and development of red mark syndrome in rainbow trout (Oncorhynchus mykiss)* Aquaculture, Volume 560, https://doi.org/10.1016/j.aquaculture.2022.738577.

4. **Orioles M.**, Bulfoni M., Saccà E., Cesselli D., Schmidt J.G., Galeotti M. (2022) *Development and application of a sensitive droplet digital PCR for the detection of red mark syndrome infection in rainbow trout (Oncorhynchus mykiss)*, Aquaculture, Volume 551, https://doi.org/10.1016/j.aquaculture.2022.737910.

**5.** Metselaar M., **Orioles M**., M. Galeotti, A. Adams, K.D. Thompson (2022) *Red Mark Syndrome – Current State of Knowledge*. Aquaculture. Volume 549, https://doi.org/10.1016/j.aquaculture.2021.737748.

**6.** M. Galeotti, G. Sarli, R. Sirri, L. Mandrioli,, P. Beraldo, P. Bronzatti, R. Giavenni, **M. Orioles**, G.E. Magi and Volpatti D. (2021) *Red mark syndrome of trout (Oncorhynchus mykiss; Walbaum, 1792): histopathological scoring and correlation with gross lesions*. Journal of Fish Diseases. https://doi.org/10.1111/jfd.13391

**7.** Galeotti M., Volpatti D., Byadgi O., Beraldo P., **Orioles M**, Sarti M., Magi GE. (2021) *Red Mark Syndrome (RMS) in farmed rainbow trout: first report of outbreaks in Bosnia and Herzegovina.* Journal of Fish Diseases. https://doi.org/10.1111/jfd.13336

# Summary & Introduction

The subject of this PhD project has been a non-lethal skin disease affecting farmed rainbow trout worldwide and named red mark syndrome (RMS). To date, the etiology of this syndrome is still unknown, but a *Midichloria*-like organism (MLO) is strongly believed to be associated with it. As reported by the results of the 25th Annual Workshop of the European Reference Laboratories for Fish Diseases (DTU-AQUA, Denmark), RMS remains to date a major concern in rainbow trout farming. This disease does not cause relevant mortality nor has any impact on growth rate, but it does cause a marked downgrade of commercial products.

The objectives of the research studies described here are to contribute to create a gold standards diagnostic approach, to explore novel diagnotics techniques, especially rapid methods for early detection, and to contribute to the definition of a causative agent for RMS. Most importantly the work developed during this PhD aimed at establishing a network of collaborative institutions and companies, both on a national and international level. Each chapter of this thesis represents the description of one of these objectives.

The first phase of this project consisted of a trial, which tested the effect of 3 temperatures on the transfer of MLO from RMS-affected fish to naïve SPF cohabitants at the DTU-AQUA (Denmark) facilities. Through electron microscopy, this study visualized for the first time MLOs in fish affected by RMS in experimental conditions. Furthermore, a strong association between the disease and the presence of MLO was established. This research project gained funds and support of Aquaexcel 2020, European Association of Fish Pathologists (EAFP), and MSD Animal Health UK.

Skin and spleen samples taken from the experimental infection were then used both to create a histological scoring system, and to develop and apply a sensitive droplet digital PCR for the detection of MLO. The histological scoring system was modified starting from the results of a

dedicated study in the field, including healing stages as well.

The development and application of a specific ddPCR technique, was possible through the cooperation between DI4A and human clinical pathology laboratory at the human hospital of Udine. This extremely sensitive molecular technique was then used to detect and quantify MLO DNA from different environmental sources like water, blood and sediment both in field and experimental cases.

Through the course of the first and second year of the PhD, the collaboration with Moredun institute of research (Scotland) and Aquatic Vets Ltd. made possible the publication of the first critical review article on RMS disease.

Experience from field cases lead to the first signalament of the disease in Bosnia-Herzegovina, whereas during 2022 an outbreak of RMS was reported in a RAS system in Slovenia. For the first time RMS was described in juvenile rainbow trout as small as 35grs and MLO DNA was extracted from water samples.

During the course of the project, we collected epidemiological data from more than 100 Italian rainbow trout farms through ad hoc questionnaire and map the presence of RMS across Italy.

The present work could represent the foundation for future studies focused towards the complete molecular characterisation of MLO. Together with this, the use of ddPCR applied to MLO DNA and environmental DNA can certainly help to implement both biosecurity measures and lead to early diagnosis or even prediction of RMS outbreaks.

Finally, studies of economic impact of this disease are needed to develop possible management strategies and options, especially from a commercial point of view.

# 1. Red mark syndrome disease: an overview of the current knowledge

## Red mark syndrome (RMS) and other skin conditions in rainbow trout rainbow trout

The occurrence of skin conditions is frequently seen in rainbow trout (*Oncorhynchus mykiss*) aquaculture. Red mark syndrome is characterised by multiple skin lesions, which can be seen over the body of the fish but are mostly found on the flank. Market size fish, usually over 100 g, are most commonly affected. Severe reddening of the skin and elevation of the lesion are typical. To date, RMS has only been identified in rainbow trout, although lesions similar to RMS have been seen in brown trout (*Salmo trutta*), wild-caught rainbow trout, sea-going rainbow trout, cutthroat trout (*Oncorhynchus clarkii*), white fish (*Prosopium williamsonii*) (Metselaar et al., 2011; Oman, 1990), sea bream (*Sparus aurata*) (Bruno et al., 2007; Zarza and Padros, 2007) and in wild-caught common dab (*Limanda limanda*) (Vercauteren et al., 2019). However, none of these cases have been confirmed as RMS, based on histology. The skin condition referred to as strawberry disease (SD), synonymous with red mark syndrome, first appeared in the USA in the late 1960s (Erickson, 1969) and Europe in the 1980s (Fleury et al., 1985). The first reported outbreak in the USA was linked to an individual batch of eggs (Nevada Department of Wildlife, 1974). It was subsequently reported in the United Kingdom (UK) in late 2003 (Verner-Jeffreys et al., 2006), where it was also believed to be linked to a single batch of eggs, imported from the USA. Red mark syndrome and SD are now regarded as the same condition (Oidtmann et al., 2013), based on 16 s PCR sequencing analysis (Metselaar et al., 2010) and the condition is referred to as RMS in the present review regardless of geographic location. Outbreaks of RMS subsequently occurred in 50% of UK rainbow trout farms, which could be linked to the first disease outbreak (Adam, 2009; Girons Ingles, 2005;

Verner-Jeffreys et al., 2006). The condition has rapidly spread to the rest of Europe (Figure 1) [Switzerland and Austria (Schmidt- Posthaus et al., 2009), France (Fleury et al., 1985), Finland (Bruno et al., 2007), Denmark (Henriksen and Schmidt, 2017), Germany (Licek et al., 2008), Spain, Serbia (Radosavljevic et al., 2009), Italy (Galeotti et al., 2011), Turkey (Kubilay et al., 2014), Slovenia (Galeotti et al., 2017b), Bosnia and Herzegovina (Galeotti et al., 2021a)] and more recently to the Middle East [Iran] (Sasani et al., 2016), the Americas [USA and Chile (Sandoval et al., 2016)], Asia [South Korea (Oh et al., 2019) and Japan (St-Hilaire and Jeffrey, 2004)].

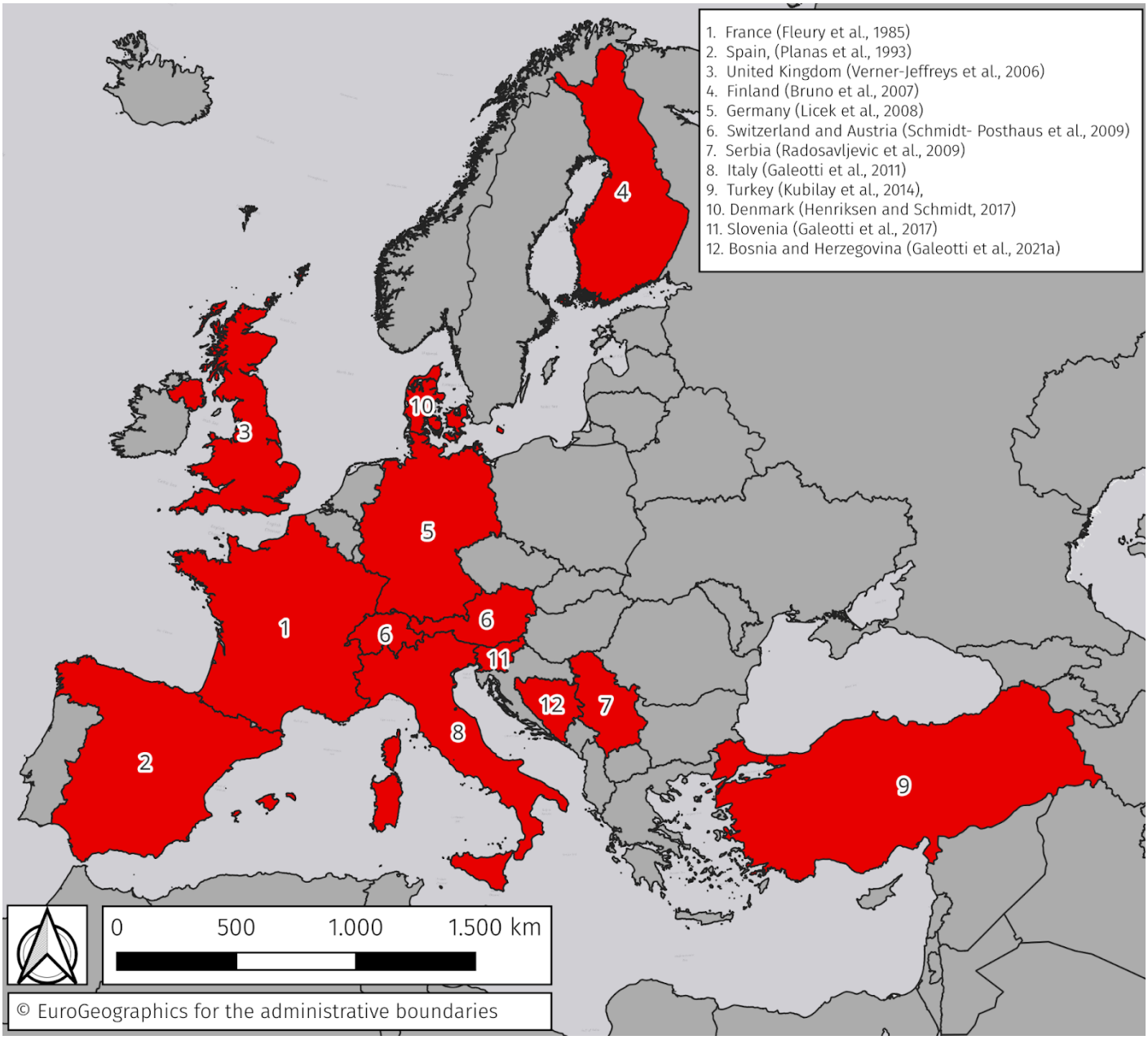


**Figure 1:** Spread of RMS in Europe. In red colour all the countries were outbreaks of the disease have been reported. France (Fleury et al., 1985), Spain (Planas et al., 1993), Finland (Bruno et al., 2007), Germany (Licek et al., 2008), Denmark (Henriksen and Schmidt, 2017), Serbia (Radosavljevic et al., 2009), Switzerland and Austria (Schmidt- Posthaus et al., 2009), Italy (Galeotti et al., 2011), Turkey (Kubilay et al., 2014), Slovenia (Galeotti et al., 2017b), Bosnia and Herzegovina (Galeotti et al., 2021a)]. More recently the disease has been described to the Middle East [Iran] (Sasani et al., 2016), the Americas [Chile (Sandoval et al., 2016)], Asia [South Korea (Oh et al., 2019)].

Apart from several case reports, no recent studies define an updated incidence of RMS and its current economic impact on trout aquaculture. The last report by Jørgensen et al. (2019) briefly described that in 2016, one third of Danish trout farmers reported incidences of the disease, but no specific data was provided. The presence of lesions does not affect the appetite or growth of the fish but does affect the fish's appearance, which can lead to economic losses for the farmer (Ferguson et al., 2006; Noguera, 2008). Generally, it is well recognized that this skin condition has proven challenging for the trout industry to deal with. More so because the fish product is downgraded due to the presence of skin lesions, resulting in economic losses for the farmer (Verner-Jeffreys et al., 2008). The manuscript by Oh et al. (2019) describes several cases of the disease in rainbow trout in South Korea, in which macroscopic lesions resembling RMS can be seen in juvenile rainbow trout weighed less than 30 g. Mortality is usually not associated with RMS, despite the high level of morbidity often associated with the disease, which can be between 5 and 50%, but has been reported to be as high as 90% during some RMS outbreaks (Noguera, 2008). The high level of mortality observed by Oh et al. (2019) does not reflect the classic case definition for RMS and further investigations are warranted to confirm the RMS status of these fish. A case definition for RMS is best described by Oidtmann et al. (2013). It defines RMS to only be present in rainbow trout of more than 50 g body weight, in freshwater of 16 ◦C and can still be present at 2 ◦C

(Oidtmann et al., 2013). It has also been referred to as cold water strawberry disease or gravel rash (Girons Ingles, 2005; Oidtmann et al., 2013). The skin lesions, which are on its flanks, are focal to multifocal, bright red in appearance, and 5 mm to several cm in diameter. Exophthalmia and skeletal deformities are mentioned by Bruno et al. (2007), but these are likely to be unrelated to RMS as these symptoms have not been reported in any later RMS-related publications or listed in the case definition (Oidtmann et al., 2013). There are some indications that the disease is seen at temperatures up to 18 ◦C (McCarthy et al., 2013), but there is no direct support for this. Most recent studies have shown that mild forms of RMS develop and in fewer fish at 19 ◦C, and at 15.5 ◦C the disease symptoms disappear more quickly compared to 12 ◦C (Orioles et al., 2021b). It is important to distinguish RMS from other similar skin conditions found in rainbow trout. Warm water strawberry disease (WWSD) is another skin condition seen in rainbow trout, but the pathology and temperature range for this condition is different from that for RMS. It is, therefore, believed that WWSD is a different skin condition to RMS based on the epidemiological and pathological characteristics of the disease (Barker and Algoet, 2000; Ferguson et al., 2006; Verner-Jeffreys et al., 2008; Oidtmann et al., 2013). There is also a condition called rash, a skin pathology only seen in the US, which might resemble WWSD, and differs from the symptoms observed with RMS (Oidtmann et al., 2013). A more recent skin condition seen in rainbow trout in the UK, referred to puffy skin disease (PSD), is of unknown causality. A case definition for PSD was made based on epidemiology, clinical signs and presentation, gross pathology, and light and electron microscopy. This skin disease, unlike RMS, affects the appetite and overall health status of the fish and can result in mortality, differentiating it from RMS (Maddocks et al., 2014).

**Aetiology**

A variety of different pathogens have been proposed to cause RMS. In 1985, an adeno-like virus was isolated from rainbow trout with RMS-like symptoms (Fleury et al., 1985). Virus particles were

observed under transmission electron microscopy (TEM) with an icosahedral symmetry containing 6 capsomeres on each side giving a total of 252 capsomeres. The virus was non-enveloped with a diameter of approximately 65 nm, which is consistent with an adeno-like virus. Parasitic, fungal, and haematology investigations were also performed by the authors, but these did not identify a causative agent. An adenovirus was again identified by Oman in fish displaying RMS lesions (1990). It has not been possible to consistently find the virus in later studies despite performing TEM studies (Girons Ingles, 2005; Verner-Jeffreys et al., 2008). Other attempts were made to culture viruses, with no cytopathogenic effect (CPE) observed (Planas et al., 1993; Metselaar, 2013). Planas et al. (1993) found two bacterial species, *Pseudomonas* spp. and *Aeromonas hydrophila* in diseased fish sampled from an RMS outbreak in rainbow trout in Spain in 1993, and although the authors concluded that these bacteria were not primary pathogens, they believed them to be associated with the disease. Subsequent studies on RMS did not identify these pathogens in diseased fish (Metselaar et al., 2010; Lloyd et al., 2008; Verner-Jeffreys et al., 2006, 2008) and this may have been an incidental finding. The response of RMS to antibiotics suggested that a bacterial agent was involved in the development of the disease (Verner-Jeffreys et al., 2008; Schmidt et al., 2021) and two bacterial pathogens have consistently been associated with RMS, *Flavobacterium psychrophilum,* and a Rickettsia-like organism (RLO), now referred to as an MLO. In the initial report identifying RMS in the UK, an association was suggested between RMS and *F. psychrophilum*, the causative agent of rainbow trout fry syndrome (RTFS) (Ferguson et al., 2006). The bacterium was detected by nested PCR in lesions on the skin and hearts of two out of three formalin-fixed wax-embedded tissues analysed from RMS-affected fish, while no association was found in three fish sampled from RMS-affected fish obtained from the USA. Heart lesions typically seen during RTFS were observed in the fish. *F. psychrophilum* was not isolated from the fish using standard bacteriological techniques (Crumlish et al., 2007). The bacterium has frequently been found in RMS- affected fish by other authors, not only in RMS lesions but also in organs and at the

back of the eye (Noguera, 2008). The bacterium has also been found in the brain and liver of fish fully recovered from RMS several months after the peak of the disease outbreak. Together with the overall clinical presentation and characteristic histopathology, it was suggested that RMS is a chronic, sub-lethal septic infection of *F. psychrophilum* and the strain involved may vary from the ones causing RTFS (Noguera, 2008). A further indication that *F. psychrophilum* could play a role in RMS comes from studies using an autogenous *F. psychrophilum* vaccine, which seemed to protect rainbow trout against RMS, with a lower prevalence of RMS in the vaccinated fish (Wallis et al., 2009). As no adjuvant-only control group was included in this trial, it was not possible to establish if the adjuvant present in the vaccine offered some protection against the disease. This was addressed in a later study in which it was shown that the vaccine adjuvant gave a significant boost to the fish's immune system, lowering the prevalence of RMS (Adams, 2017). No subsequent studies have found a clear association between *F. psychrophilum* and RMS. Other studies have shown that *F. psychrophilum* is present in both RMS-affected fish and naïve fish (Jørgensen et al., 2019; Metselaar et al., 2020). Verner-Jeffreys et al. (2006) obtained a diverse range of bacteria, including *F. psychrophilum,* from RMS-lesions using standard microbiology and 16S rRNA gene libraries, none of which could be related to RMS. Schmidt-Posthaus et al. (2009) were also unable to culture *F. psychrophilum* from RMS-affected fish in Switzerland and Austria. Metselaar et al. (2020) found no association between *F. psychrophilum* and RMS when screening skin and organs from RMS- affected and naïve fish using a PCR for *F. psychrophilum*. They showed that the bacterium was present on the skin of some fish with RMS, but it was also present on unaffected fish using IHC with rabbit anti-*F. psychrophilum* serum (Metselaar, 2013). The bacterium can be found on some fish with RMS, and there is still some debate about the role of *F. psychrophilum* in the disease. Koch's postulates have never been fulfilled for its role in RMS. *F. psychrophilum* can be found in 48% of Rainbow trout farmed in Finland, without showing any signs of RMS (Bruno et al., 2007). *Flavobacterium columnaris* and *Flavobacterium* spp. were found at a later stage in the disease and were not associated with the onset of RMS and therefore were not thought to be

primary pathogens for the disease (Fleury et al., 1985). The initial report by Ferguson et al. (2006), stated that the lack of *F. psychrophilum* in affected fish from the USA was due to it being a different disease. With the knowledge that SD and RMS are the same disease (Oidtmann et al., 2013), this would suggest that *F. psychrophilum* is not the cause of the disease, but rather an incidental finding. Around the same time as the work suggesting a correlation between the presence of *F psychrophilum* and RMS lesions, the presence of the RLO was found in RMS-affected fish based on a partial 16S rRNA sequence (Lloyd et al., 2008). Lloyd et al. (2008), identified a partial 16S rRNA sequence of the MLO in 16S from rRNA libraries and developed a nested PCR (Lloyd et al., 2008) and subsequently a qPCR (Lloyd et al., 2011). More recently, the RLO identified by Lloyd et al. (2008), has been identified as a Midichloria–like organism (MLO), belonging to the family Midichloriaceae within the Order Rickettsiales (Montagna et al., 2013; Cafiso et al., 2016), and therefore the RLO is referred to as an MLO in this review, irrespective of the nomenclature used in the original manuscript. In initial studies on RMS in the UK, Verner-Jeffreys et al. (2008) also used a culture-independent bacterial 16S rDNA library approach to identify the agent associated with RMS; the authors also found the MLO sequence reported by Lloyd et al. (2008) but did not associate it with the disease (Adams, 2017). This is likely because the primers used Verner-Jeffreys et al. (2008) had mismatches for the MLO sequence (3 at the end of 5′ _end of the forward primer and 2 on the reverse primer) compared to those used by Lloyd et al. (2008). Using the primers designed by Lloyd et al. (2008), Metselaar et al. (2010) detected the MLO 16S rRNA sequence in RMS-affected fish in the UK and USA, suggesting that the same aetiological agent was responsible for RMS in both geographical regions. Metselaar et al. (2020) showed there was an association between RMS and the MLO using samples from RMS–affected and unaffected rainbow trout from the UK and the USA, with naïve fish testing negative for the presence of MLO DNA, while all fish with RMS were positive for MLO DNA. Cafiso et al. (2016) and Metselaar et al. (2020) both showed that the MLO is not only located in skin lesions, but is detected in various

organs, and in skin sections that do not present any pathological changes. These results were supported by IHC, in which antibodies specific against *Pisckirickettsia salmonis* were used to detect what was thought to be the MLO in RMS–affected fish. This method stained all cases of RMS-affected tissue, while only 5 internal organs from 22 RMS-unaffected fish were stained very weakly with these antibodies. However, no bacteria were seen in the tissue positively stained with the antibody and it was not confirmed if the anti-*Piscirickettsia salmonis* antibodies bound to epitopes common with the MLO (Metselaar et al., 2010). The antibodies against *P. salmonis*, should not be considered specific as this bacteria does not belong to the order Rickettsiales. It is a Gammaproteobacteria and not an Alphaproteobacteria. Most recently, Orioles et al. (2021a) developed and applied a specific and highly sensitive digital droplet (dd) PCR method for the detection and absolute quantification of MLO in spleen samples of fish affected by RMS. Even though MLOs are intracellular organisms are large enough to be visualised using conventional light microscopy, the MLO has only recently been seen in fresh spleen smears of RMS-affected fish (Galeotti et al., 2011), and it has been possible to visualize rickettsia in tissues of fish that were positive for the MLO by PCR using Macchiavello's stain (Galeotti et al., 2021a). The fact that the MLO was not visualised by histology in earlier studies is not unique to RMS, as this was also the case for *Francisella noatunensis* subsp. *noatunensis* infecting Atlantic cod (*Gadus morhua*) (Zerihun et al., 2011). Ultrastructural features of the MLO in RMS-affected tissues (*i.e.* skin and spleen) have been seen in field cases (Galeotti et al., 2016; Galeotti et al., 2017a, 2017b), complementing the PCR findings. The microorganisms appear oval or as a short rod, with a variable size ranging from 400 to 1000 nm in length and 100 to 500 nm in width. They have an electron-dense cytoplasm with the presence of a nucleoid and a cell wall arranged in a distinct tri-laminar structure characteristic of Gram-negative bacteria. Microorganisms visualised within the spleen were often seen in erythrocytes or macrophages within vacuoles, rarely delimitated by a distinct cell membrane and did not appear to have a clear cell wall (Galeotti et al., 2017a, 2017b). In a recent study by Oh et al. (2019), a microorganism similar to that described by Galeotti et al. (2017a,

2017b) was observed with a tri-laminar wall structure. Despite having a similar cellular membrane structure and dimension, other features typical of Rickettsiales (Fritsche et al., 1999; Sacchi et al., 2004; Sassera et al., 2006; Vannini et al., 2010; Szokoli et al., 2016) were not mentioned, such as intracellular localization, absence of a distinct host membrane around the bacteria and the presence of a halo zone (the electron-lucent layer around the bacterium). However, the morphology of the MLO described in both reports resembled bacteria belonging to order Rickettsiales. This should be confirmed with more specific methods, such as *in situ*-hybridization, immunogold staining or IHC using an antibody specific against the MLO once developed. It seems important to note that the role of ultrastructural observations remains purely descriptive for the presence of MLO and its role in the infection process has yet to be defined. The MLO maybe a secondary pathogen as it is detected by IHC, PCR, and qPCR when the lesions are already evident on the fish, however, this is less likely as the pathogen has been consistently found in and associated with RMS-affected fish in at least three different geographical areas. Generic approaches to culture the causative agent have been attempted by various authors on various agars. Solid agars have been successfully used to support the growth of intracellular organisms such as *P. salmonis* (Yanez et al., 2013) and Francisella spp. (Ottem et al., 2007), several of which have been tried unsuccessfully (Metselaar, 2010). When Verner-Jeffreys et al. (2008) cultured swabs from lesions and other organs of RMS-affected fish, was particular bacterial strain/species cultured seemed to be associated with affected tissue. Verner-Jeffreys et al. (2008) and Metselaar et al. (2020) tried to culture swabbed from RMS lesions on tryptic soy agar (TSA), Veggietone Agar, and Austral-TSFe, unsuccessfully, although lesion swabs may be an unsuitable sample because of the presence of other bacteria. With qPCR data showing the presence of the MLO in internal organs, isolation of the MLO from internal organs may be more appropriate. Specific insect cell lines, such as '*Spodoptera frugiperda* cell ine'(SF-21), 'Chinook salmon, *Oncorhynchus tshawytscha* embryo cell line' _(CHSE-214), Atlantic salmon, *Salmo salar* kidney cell lines (ASK), and Atlantic salmon, *S. salar* head kidney 1 cell line (SHK-1), successfully

used to culture *P. salmonis* (Birkbeck et al., 2004) and *Francisella* sp. (Nylund et al., 2012), were unsuccessful to culture the MLO in RMS (Metselaar, 2013). Fleury et al. (1985) used 9-day embryonated eggs to identify the Adenovirus, with no mention of bacterial infection. Injection of wax moth (*Galleria mellonella*) larvae and collection of haemolymph was tried by Carpentier (2016) without success. Until now Midichloriaceae remain uncultivable micro-organisms. Because the rickettsiae are obligate intracellular bacteria of eukaryotic cells, their cultivation requires viable eukaryotic host cells for successful culture. Strides have been made for solid agars that will support intracellular organisms such as *P. salmonis* (Yanez et al., 2013) and Francisella spp. (Ottem et al., 2007), several of which have been tried unsuccessfully (Metselaar, 2010). The most suitable time to sample fish for culture may be possible be elucidated from the infection dynamics determined from recently developed experimental infection models.

**Gross pathology and scoring systems**

RMS lesions tend to be graded based on their severity. The first documented scoring system was described by Metselaar (2010), who identified early-stage lesions as Grade 1 (Fig. 1a), appearing as small spots covered with mucus. The characteristic redness associated with RMS can be seen in the centre of Grade 2 lesions (Fig. 1b), while Grade 3 lesions (Fig. 1c) are more extensive than Grade 2 lesions, covering more surface area of the fish. Other studies have reported other types of macroscopic classification. McCarthy et al. (2013) defined the lesion stage as ‘early’, ‘mid’, ‘advanced’ _or ‘healing’, similar to Metselaar (2010). Schmidt et al. (2021), on the other hand, had a 2 fold scoring system: 0 for the presence of lesions; and 1–3 for active, healing, and healed lesions, respectively. Score 1 was sub-divided into early/mild, medium, and severe/peak. They used this scoring system for RMS cases derived from experimental infections, which was useful for reflecting the progression of lesion severity from lesion onset to healing. Orioles et al. (2019) proposed another scoring system based on both macroscopical and microscopical classifications,

divided into mild, moderate, severe, and regenerative scores. A detailed and reproducible classification for gross lesions has recently been proposed by Galeotti et al. (2021b) using five macroscopic parameters; size, colour variation, exudate, visible scales, and erosion. According to their macroscopic appearance, the lesions are classified as mild (type I), moderate (type II), or severe (type III) (Table 1 and Fig. 2). In this case, the classification system was used on fish derived from field cases and considered only the increase of lesion severity. It would appear that there is a wide consensus for the use of mild, moderate, and severe stages to define the severity of RMS lesions. Scale loss, the intensity of hyperemia/redness, and elevation of the lesion are by far the most important features to consider. It is certainly relevant to recognize regenerative stages as well, as this could provide essential practical knowledge for the farmer, regarding the healing status of the lesion. As mentioned earlier, the most accurate grading systems in terms of classifying lesion progression are certainly the ones developed from experimental infection trials, such as the ones reported by Schmidt et al. (2021) and Orioles et al. (2019, 2021b). In the authors' experience, there may be some discrepancies in defining the severity of the lesions between samples from the field and experimental cohabitation models, as the former may express the disease with more pronounced clinical signs. Ideally, gross lesions should be correlated to histological classification, but so far only one study has found a correlation between a single histological scored parameter and the three types of gross lesions (Galeotti et al., 2021b) (discussed later in the review). Finally, veterinary pathology terminology used to describe different skin lesions and progression of lesions should be precise and standardised. Simple terms such as "red spots" _could lead to a misleading interpretation of gross pathology findings.

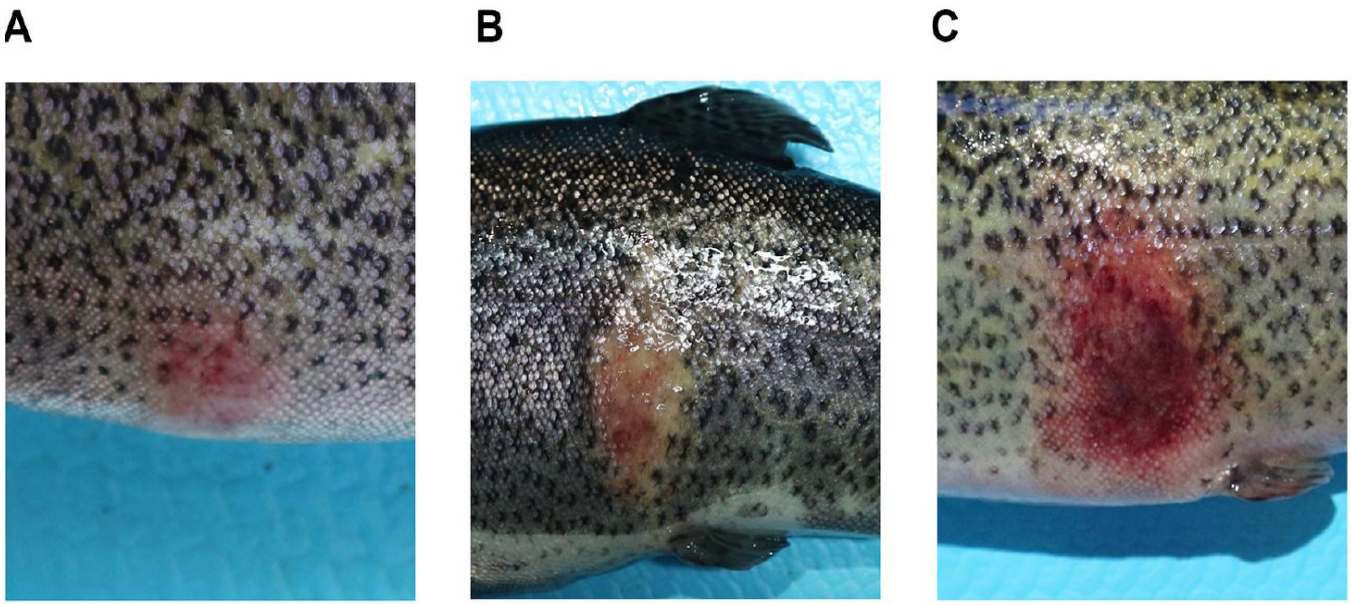



**Figure 1:** Classification of RMS skin lesion by macroscopic features.

a) Type I lesions consisting of small flat macula, slight redness, and visible scales
b) Type II lesions characterized by slightly raised patches, with the largest of about 2 cm covered by clear exudate associated with multifocal petechiae and mild scale loss.
c) Type III lesions show slightly raised patches, more than 2 cm, covered with marked redness, hemorrhages and scale loss.

**Histology and scoring systems**

Histologically, RMS has been described both in the field and experimental settings (Ferguson et al., 2006; Verner-Jeffreys et al., 2006; Noguera, 2008; Oidtmann et al., 2013; Orioles et al., 2019; Jørgensen et al., 2019; Galeotti et al., 2021a; Orioles et al., 2021b), and there have been attempts to standardise the histological classification and scoring of RMS lesions (McCarthy et al., 2013;Galeotti et al., 2013); Schmidt et al., 2018; Jørgensen et al., 2019; Orioles et al., 2019; Galeotti et al., 2021b). McCarthy et al. (2013) described a scoring system based on the histological appearance of the lesion by comparing the relative level of infiltration, disruption of the stratum

compactum, and scale resorption/ regeneration ranging from normal skin (severity: 0) to advanced lesions (severity: 10). The most recent classification of skin lesions based on histology describes a progressive loss of epithelium and scale pockets, progressive recruitment of inflammatory cells into the stratum compactum of the dermis, loss of regular architecture of the stratum compactum, and an increase in granuloma-like reactions surrounding the small vessels, nervous fibres of hypodermis and in the muscular fibres, which were statistically positively correlated with the progression of macroscopic lesions (Galeotti et al., 2021b). The initial cellular inflammatory reaction observed in RMS seems to target the scale pocket and the dermis spongious area, then moves progressively to the hypoderma, and in severe cases the dermis compactum, epidermis, and muscular layers (Noguera, 2008). There is a large infiltration of inflammatory cells, such as lymphocytes, neutrophils, and macrophages, so much so that the lesions appear raised compared to normal skin, and severe loss of architecture is observed. Loss of scales is commonly seen at the lesion site, possibly due to the large infiltration of neutrophils and oedema inside and around the scale pockets (Ferguson et al., 2006; Noguera, 2008; Verner-Jeffreys et al., 2008; Schmidt-Posthaus et al., 2009). Neutrophils in this area have not been reported in subsequent studies (Galeotti et al., 2011; Oidtmann et al., 2013; McCarthy et al., 2013; Sasani et al., 2016; Sandoval et al., 2016; Orioles et al., 2019; and Galeotti et al., 2021b), where all stages of lesion development appear as a lymphocytic infiltration into the stratum spongiosum, immediately surrounding the scale pocket. Scale reabsorption and regeneration are also often observed. The presence of multinucleated giant mesenchymal cells, resembling osteoclasts, is a very common finding, especially in severe lesions, where scale reabsorption is observed (Fig. 3) (Oidtmann et al., 2013; McCarthy et al., 2013; Orioles et al., 2019; Galeotti et al., 2021b). Red mark syndrome has limited epidermal involvement and extensive lymphocytic infiltration into the sub-dermal layer, with full-thickness dermatitis (Verner-Jeffreys et al., 2008). Within established RMS lesions, neutrophils can be seen in connective tissues between the epidermis and dermis, and between the dermis and subcutis. Pathology is not

exclusively seen in the skin in RMS; changes are also noted in the liver, kidney and spleen, as well as exophthalmia, myocarditis and skeletal deformities (Bruno et al., 2007). Myocarditis has been reported in 20% of the RMS-affected fish also showing cardiac pathology (Ferguson et al., 2006), with acute necrotising myocarditis seen in smaller fish (Noguera, 2008). More recently, Galeotti et al. (2021b) described hyperplasia of the white pulp with perivascular histiocytic cell proliferation in the spleen, lymphocytic vasculitis in the liver and mild vacuolar degeneration of tubular epithelium in the kidney. Ulceration of the skin has also been described in RMS (Verner-Jeffreys et al., 2006; Orioles et al., 2019), although some authors think this is due to the presence of secondary pathogens (Ferguson et al., 2006) and is not included in the case definition (Oidtmann et al., 2013). Considering the inflammatory response observed in very early-stage lesions, not including the epidermis, it has been suggested that the disease occurs from an internal infection and the external lesions that are observed are thought to be the result of a hypersensitivity reaction by the fish's immune response to an organism (Barker and Algoet, 2000; Ferguson et al., 2006; Noguera, 2008; Verner-Jeffreys et al., 2006), although this type of reaction could be triggered by other factors, such as changes in water quality or temperature and/or stress following husbandry procedures, such as grading and handling. It is still unclear what the exact trigger for this reaction is, and due to the long incubation period before lesions occur this may be difficult to determine. Establishing a reproducible experimental challenge model as discussed below (Jørgensen et al., 2019; Schmidt et al., 2021), will help to understand the disease process involved in RMS. During disease development, Jørgensen et al. (2019) characterized local cellular immune responses and regulation of immunologically relevant genes in the skin of the cohabitants by IHC and qPCR, respectively. These results again support the MLO as the causative agent of RMS and fish overcome the infection by a Th1-type response supplemented by a possible T cell-independent production of antibodies. Furthermore, Galeotti et al. (2021b) recently suggested that the presence of micro thrombosis and epidermal necrosis observed in more advanced lesions is similar to those seen in diseases like canine scabies, where the accumulation of immunocomplexes (type III

hypersensitivity immune response), can coexist with delayed-type hypersensitivity (type IV). These observations would further support an immunopathological mechanism. The findings of McCarthy et al. (2013) are supportive of this, describing the immune response in RMS-affected fish in detail. In the same publication, they also reject the hypersensitivity theory as these types of reactions are often recurring or chronic, which is not consistent with the single, non-recurring characteristics of an RMS outbreak. The MLOs can be visualised using conventional light microscopy, especially with special stains for MLOs like Macchiavello's stain. So far the MLO has only been observed once in fresh spleen smears of RMS-affected fish (Galeotti et al., 2011). Observing the MLO can be particularly challenging, and may have been overlooked in early RMS studies due to the small size of the organism and the fact that its presence had not yet been linked with the infection. Regardless of the histological classification proposed, it may be important to standardise this based on similar criteria used for scoring gross pathology lesions *i.e.* cases derived from cohabitation infections may reflect more accurate ways to assess the progression of the disease over time, showing very early and less dramatic histological signs and regenerative cases, which are not evaluated in field cases. Given the uncertainty of the aetiology of RMS, the qPCR used to detect and quantify the MLO, may not be a suitable gold standard for RMS diagnostics. Although a correlation between histological grades of severity and gross pathological findings remains to be determined, histological examination remains essential for defining disease progression.

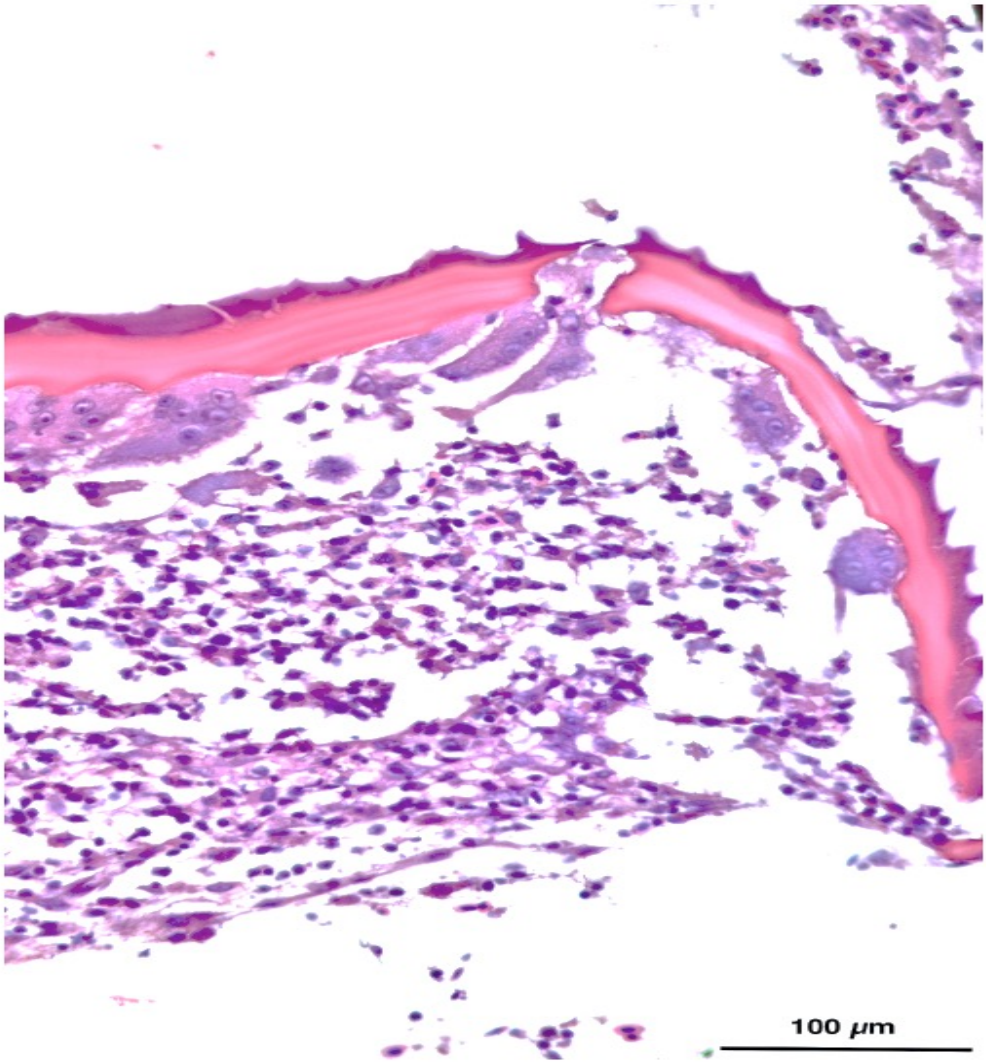



**Figure 3:** Lesion type 2, with numerous multinucleated osteoclasts in phase of scale re-absoprtion

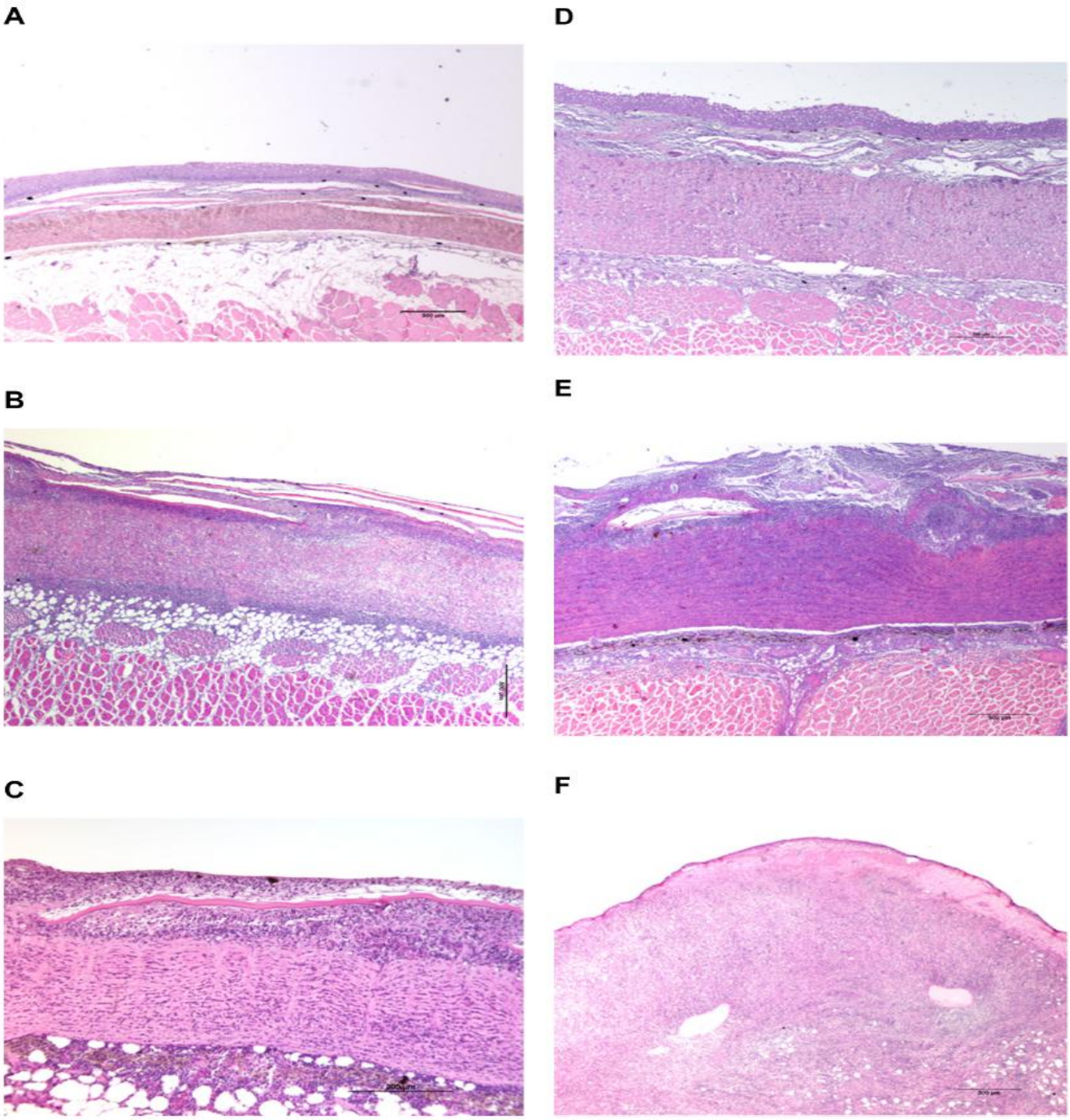



**Fig. 2:** Histological features of the three types of RMS lesions. a) Histological features of the **Type I lesion**, revealing a slight inflammation involving all the skin layers, including the hypodermis. The epidermis is intact. The scale pockets and scales are present, with a slight cellular infiltration (H&E). b) Histological features of the **Type II lesion**, revealing an inflammatory response involving all the skin layers from epidermis to hypodermis, until the underlying muscular tissue.

The *stratum spongiosum* appears moderately infiltrated, mainly in the areas below the scales, which are still intact. The scale pockets and scales are present, but the cellular infiltrate is more consistent The scales begin to show re-absorption phenomena. The *stratum compactum* of the derma appears thickened with a moderate inflammatory infiltrate, recruited also towards the hypodermis and the muscular layer (H&E). c-d) Histological picture similar to figure b, of type II lesions. e-f) Histological features of the Type III lesion showing a severe cellular infiltration involving all the layers from epidermis down to the muscular tissue with epidermis partially missing. e) The *stratum spongiosum* is thinned and no scales can be observed Some scale pockets and scales are still present, but the cellular infiltrate is severe The dermal *stratum compactum* appears thickened and severly infiltrated, deeply modifying the layer's architecture. Severe infiltration reaches the hypodermis (panniculitis) and the underlying muscular layers (myositis), spreading also between the myosepta (H&E). f) In this lesion the infiltrate is very severe in all layers. It is not possible to highlight the limits between *stratum spongiosum* and *stratum compactum* that appear very thick. Scale pockets and scales they have completely disappeared.

**Transmission studies**

Red mark syndrome is transmittable through cohabitation (Erickson, 1969; Oman, 1990; Verner-Jeffreys et al., 2008; Metselaar et al., 2010; Jørgensen et al., 2019). Recently developed co-habitation models further support the involvement of the MLO in RMS and have provided valuable insights into the development of RMS symptoms and treatment (Jørgensen et al., 2019; Schmidt et al., 2021; Pasqualetti et al., 2021; Orioles et al., 2021b). Verner-Jeffreys et al. (2008) at Centre for Environment, Fisheries and Aquaculture Science (CEFAS), UK and more recently Jørgensen et al. (2019) at the Technical University of Denmark, National Institute of Aquatic Resources (DTU Aqua) artificially transmitted RMS through cohabitation infections, repeating the results from an earlier American study with RMS (Erickson, 1969). Metselaar (2013) also obtained RMS-like

lesions when cohabiting RMS-affected fish with naive fish, but the results were inconclusive. Experimental designs for such infection studies includes the use of specific-pathogen-free (SPF) or naïve fish, reared in trial facilities or from eggs purchased from certified commercial hatcheries, and placed together with cohabitants showing characteristic RMS lesions (seeders). This has been described in detail by Jørgensen et al. (2019) and Schmidt et al. (2021), for their co-habitation studies, where rigorous health checks were performed before starting the trial. The cohabitation was either direct, sharing the same tank, or indirect using mesh screens to separate groups of fish within the same tank. Strict biosecurity is necessary to minimize the risk of transferring potentially infectious material between tanks, such as the use of lids, dedicated nets, separate cleaning equipment, and appropriate staff training. In the study of Jørgensen et al. (2019), the fish had both *F. psychrophilum* and *Ichthyophthirius multifillis* infections during the cohabitation trial, leading to some fish having to be euthanatized. For co-habitation trials reported in the literature, municipal water in recirculating systems (with filtration and UV disinfection) or dechlorinated laboratory water flow-to-waste systems have been used (Verner-Jeffreys et al., 2008; Jørgensen et al., 2019). Water temperature was generally maintained at 10–12 ◦C and fish were kept in variable-sized tanks, from 180 L to 300 L, made of different materials, such as cylindrical clear plastic (PETG), Fish were provided with a standard 12:12 h light:dark cycle and fed with a standard commercial diet based on body weight. Passive integrated transponder (PIT) technology was most commonly used to identify individual fish for assessing disease progression at an individual level. Cohabitation studies have been used to investigate the role of the MLO in the disease process. Verner-Jeffreys et al. (2008) examined the early stages of RMS in two cohabitations studies. In the first study, forty 75 g naïve rainbow trout, obtained from a hatchery with no previous history of RMS and raised in dechlorinated water at 10 ± _0.5 ◦C, were cohabited with fifteen RMS-affected fish (average weight 410 g) separated by a mesh screen. The affected fish came from two pre-selected farms, with on-going RMS outbreaks with fish showing typical RMS lesions. In a second trial performed at 10 ±

_0.5 ◦C, naïve fish were cohabitated for 62–72 days with the RMS-affected fish, again separated by a mesh screen, but this time they were maintained for 104 days. In both studies, the first signs of RMS were observed after 650 DD, indicating that the disease takes a long time to develop (Verner-Jeffreys et al., 2008). Schmidt et al. (2021) subsequently showed from their transmission trials that the onset of the disease started to occur approximately 660 degree days (DD), consistent with other studies (Erickson, 1969; Oman, 1990; Metselaar, 2013). At the end of the study, at 730 DD, Verner-Jeffreys et al. (2008), found 124 of the 310 surviving fish (40%) had typical RMS lesions. More recently, in the cohabitation model established at DTU-Aqua, Denmark, described by Jørgensen et al. (2019), peformed12◦C, lesion severity peaked after 420 DD after which inflammation resolved and healing had started to take place. They were able to tract the MLO, which was no longer detectable in fish once healing had started. More than 20 batches of specific pathogen-free fish have now been infected using this model, in which fish consistently develop signs of RMS (Schmidt et al., 2021). Overall, the results strongly suggest the involvement of an infectious agent, such as the MLO. A recent study by Pasqualetti et al. (2021) suggested that *Ichthyophthirius multifiliis* may act as a vector for MLO transmission. This was tested by infecting RMS-diseased rainbow trout with *I. multifiliis* theronts, and subsequently investigating the presence of RMS-MLO in tomonts detached from the fish. Real-time PCR analyses clearly showed that *I. multifiliis* previously exposed to RMS-affected fish tested positive for the MLO, suggesting that the bacterium can at least be transiently acquired and carried by the protozoan. Moreover, statistical analyses suggested a possible level of vertical transmission in *I. multifiliis* from one trophic stage to the next. Red mark syndrome is seen at water temperatures below 16 ◦C, and although lesions have been seen at higher water temperatures, it is unclear if RMS develops at higher temperatures rather than just manifesting at this temperature due to the long latency period required for the disease to develop (Metselaar - personal observation). The lesions tend to subside at higher water temperatures, and the healing time of lesions is associated with water temperature (Ferguson et al., 2006). The recent co-habitation study by Orioles et al. (2021b) followed the development of RMS at three different

temperatures (12 ◦C, 15.5 ◦C, and 19 ◦C) for 11 weeks. The severity of lesions peaked at 11 weeks from the onset of the trial at a standard 12 ◦C, whereas the course of the disease was faster at 15.5 ◦C, where the disease peaked at 8 weeks and completely resolved at 11 weeks. At 19 ◦C few fish developed a mild form of RMS with a peak seen at 8 weeks.

**Epidemiology**

Since its first appearance in the USA in 1946, the spread of RMS through the UK, Europe, and further afield has been relatively fast, highlighting the need to understand why this has happened. The biggest risk to biosecurity on any farm is always the importation of livestock. One risk factor associated with the development of RMS is thought to be the supply of fish and eggs, with an increased risk of a farm developing RMS if it obtains fry from more than four different suppliers (Adam, 2009). Increasing biosecurity to either only import eggs from RMS-free sources or having in-house broodstock may help reduce such risks. Fifty percent of rainbow trout farms in the UK are not affected by the disease, and these tend to be smaller, closed farms. A closed farm where no fish are imported and movements of fish are restricted (*i.e.* from broodstock to the hatchery and then to on-growing sites) will be less likely to contract new diseases such as RMS. A further challenge is the presence of wild stock swimming freely in the waterbody. A single company having risk. Epidemiological studies have shown that once RMS is observed on a farm, 10–60% of fish will develop clinical signs of RMS 2–3 weeks later (Verner-Jeffreys et al., 2006). A rolling infection is then observed in the population, with some fish starting to show healing of lesions, while other fish are just starting to develop RMS symptoms. Red mark syndrome has also been seen in fish in seawater, but always shortly after transferring the fish to sea, therefore is thought to have originated in freshwater (Metselaar - personal observation). It was noted that no reoccurrence of disease outbreaks occurs in the same batch once the fish have recovered (unknown, Red Mark Syndrome

(RMS) Meeting, 2009). Treatment was possible with broad-spectrum antibiotics such as oxolinic acid, oxytetracycline, and florfenicol. Disinfectants like chloramine T and 10% neutral buffered formalin were able to clear the condition before harvest but were less effective compared to antibiotics (Robert Huges,Red Mark Syndrome (RMS) Meeting, 2009; Schmidt et al., 2021). Horizontal transmission by direct or indirect contact is supported by the cohabitation studies. Vertical transmission could be possible as it was suggested batches of eggs were responsible for initially introducing the disease (Nevada Department of Wildlife, 1974; Verner-Jeffreys et al., 2006). As egg transfers across borders are very common, and RMS has only spread relatively slowly, the role of vertical transmission needs to be investigated further. No difference was found in the mean water temperature between infected and control farms (Adam, 2009). Orioles et al. (2021b) showed that disease progression is faster at higher temperatures. Adam (2009) showed that there might have been two separate introductions of infected fish, one in 2003 and another in 2006 when there was a steep increase in the number of newly infected farms. Potential risk factors for the development of RMS include all mechanical methods of handling fish. Therefore farms at higher risk of developing RMS should keep mechanical handling to a minimum and implement "old-fashioned good husbandry", where actions are aimed to reduce stress on the fish. However, many factors that induced stress did not appear to elevate the risk of fish developing RMS, while hand netting and handling lowered the risk. Sourcing fish from more than four suppliers was also found to increase the risk of fish developing RMS. The number of fish deliveries to a farm or connections through the river network did not seem to increase the farmer's chances of developing the disease, while the movement of fish or ova onto the site appeared to be the most important route for introducing the disease. This is supported by the fact that large-scale production sites have a higher likelihood of becoming infected than smaller producers (Adam, 2009). In conclusion, the severity of an infection is dependent on several factors and RMS is noted to be multifactorial, where certain farming practices and environmental factors make some stocks of fish more susceptible to the condition (Bruno et al., 2007; Botelho-Nevers and Raoult, 2011). There is some speculation of the

current phylogeny of Midichloriaceae. Two different groups of protists, ciliates (Alveolata) and Acanthamoeba species (Amoebozoa) may be particularly relevant hosts for the MLO (Serra et al., 2015). Aquatic eukaryotic single-celled amoeba, inhabiting intake water, could act as a vector/reservoir for the midichloriacea associated with RMS. In this context, the use of novel PCR methods, such as droplet digital PCR used by Orioles et al. (2021b), with high positive predictive values and reproducibility, may be very useful, especially to investigate the presence of MLO in possible vectors such invertebrates and parasites, water, eggs, and environment in general, where pathogen DNA can be scarce. Pasqualetti et al. (2021); as mentioned above *I. multifiliis* has also shown to be a potential vector. Surveillance of disease and more data in the current incidence of RMS in Europe and worldwide should be implemented through large-scale studies combining data from different institutions. Monitoring pathogen levels in water samples could aid early detection and surveillance especially in RAS systems and for research purposes.

**Treatments for RMS**

If left untreated, RMS-lesions will resolve without leaving any notable marks on the fish and does not cause mortalities. There are some reports of systematic fallowing and disinfection of ponds helping to eradicate RMS from the farm (Adam, 2010; Metselaar, 2010). Moving fish to warmer water also results in more rapid recovery (Bruno et al., 2007). RMS has been considered to be responsive to antibiotic treatment from earlier studies (Ferguson et al., 2006; Verner-Jeffreys et al., 2008). Only a recent study investigated the effectiveness of florfenicol, oxolinic acid, and oxytetracycline against RMS, using a cohabitation model to transmit the disease, has given scientific proof for the effectiveness of antibiotic treatment fish (Schmidt et al., 2021). It concluded that these antibiotics effectively reduced the amount of MLO present in the skin of affected fish, assessed by qPCR. Furthermore, they also reduced the RMS-like skin lesions in the experimentally

infected fish. Although it is possible to treat RMS with antibiotics and disinfectants, which help to clear the fish of RMS lesions before harvest, there are major drawbacks with the use of antibiotics. There is a required withdrawal period before harvest and an additional cost associated with the treatment itself. There is currently no official treatment recommended to reduce RMS symptoms (Schmidt et al., 2021), although oxytetracycline is most commonly used in the USA (Metselaar, personal comment). Other treatments that have been used include Branzil (oxolinic acid), Florocol (florfenicol), chloramine T, sodium chloride 3 ppt bath, functional feeds (*e.g.* Skretting response diet), formalin or mild stress (*e.g.* grading) (Pond, 2006; Verner-Jeffreys et al., 2008). Topical treatment with antibiotics has also been occasionally used (Terramycin®, Pfizer) (Metselaar - personal observation). Although it is possible to treat RMS with antibiotics and disinfectants, which help to clear the fish of RMS lesions, there are major drawbacks with the use of antibiotics. As lesions tend to appear in near market size fish the required withdrawal period, and the additional cost associated with the treatment itself, are preventing treatment in most cases. There are also ethical concerns about using antibiotics to treat RMS because of their environmental impact. This has to be set against a disease that is not lethal with a seemingly low impact on fish welfare. Furthermore, the use of antibiotics in organic trout farming may be restricted. In the USA, where withdrawal periods are not linked to water temperature, the application of antibiotics is not as restrictive and are therefore more commonly used there to treat RMS (Metselaar - personal observation). Culturing MLO and its isolation are certainly the first steps in the development of an effective treatment strategy through vaccination. Unfortunately, this has been unsuccessful so far. Future studies may look into prevention strategies rather than further treatment options. The exposure of fingerlings and juvenile trout to RMS to trigger the development of natural immunity has not been reported in the literature. Even though more than one exposure may be necessary to maintain effective levels of immunity, this strategy may be a valid option considering that the disease does not generally develop in juvenile trout.

## Conclusions

Red mark syndrome is and might remain of unknown causality. The strongest candidate remains the MLO as all other possible agents mentioned lack sufficient evidence to associate them with RMS. In particular, no association was found between the RMS-affected fish and *F. psychrophilum* other than superficial presence in the skin, indicating its presence as an incidental finding. Working with intracellular pathogens like the MLO has several challenges. To fulfil Koch's postulates or develop preventive measures such as a vaccine, it is usually necessary to isolate the causative agent. So far, all attempts to isolate a bacterium, using several different methods, have failed (Erickson, 1969; Fleury et al., 1985; Noguera, 2008; Olson et al., 1985; Oman, 1990; Planas et al., 1993; Schmidt-Posthaus et al., 2009; Verner-Jeffreys et al., 2006; Verner-Jeffreys et al., 2008). Although selective media can be chosen for the suspected causative agent, it does not always guarantee the growth of the bacterium. So far, all attempts to isolate a bacterium, using several different methods, have failed (Erickson, 1969; Fleury et al., 1985; Noguera, 2008; Olson et al., 1985; Oman, 1990; Planas et al., 1993; Schmidt-Posthaus et al., 2009; Verner-Jeffreys et al., 2006; Verner-Jeffreys et al., 2008). Future research should focus on confirming the causality of the MLO in RMS, MLO culture and prevention strategies. The point of infection is likely to be before the lesions appear. Future research should focus on that latency period of the disease around 650 DD. This is best done using diseases models, such as the one developed by Jørgensen et al. (2019), and which will inform on how disease progression relates to RMS pathology. The use of novel, highly sensitive and specific techniques, such as digital droplet PCR will allow infection dynamics to be monitored more accurately. This will also inform when best to sample fish to attempt pathogen isolation. As mentioned earlier, a hypersensitivity reaction by the host to the MLO is one hypothesis for the development of RMS. Understanding the factors that cause RMS, as well as trying to isolate the MLO, are essential for developing more targeted solutions for the control of RMS. Such strategies

could focus on helping to build natural immunity of the host against RMS to avoid the use of antibiotics. The risks involved in fish developing RMS are thought to be multifactorial, where certain farming practices and environmental factors make some stocks of fish more susceptible to the condition (Bruno et al., 2007; Botelho-Nevers and Raoult, 2011). The role of water, eggs, environment, invertebrates and parasites as possible MLO vectors certainly requires more investigation. Finally, it is important to collect up-to-date data about the incidence of RMS to be able to determine its economic impact on trout aquaculture, more accurately. Surveillance of disease and more data on the current incidence of RMS in Europe and worldwide should be implemented through large-scale studies combining data from different institutions. Monitoring pathogen levels in water samples could aid early detection and surveillance especially in RAS systems and for research purposes. This information could put RMS under the spotlight and increase interest in research related to this disease.

# 2. A questionnaire-based survey on the presence of red mark syndrome in Italian rainbow trout (oncorhynchus mykiss) farms

## 1. Introduction

Existing epidemiological data of RMS consist of several reports of outbreaks without any structured study estimating its prevalence at a national or European level. Therefore, the evidence of the current economic impact of RMS on trout aquaculture remains unknown, but it is well-recognized as challenging for the industry (Metselaar et al., 2022). In particular, producers farming portion-size trout (300-500g) and organic seem to be more affected by RMS. In these production settings, the impact of RMS has been considered high, with more than 5% of their production costs attributed to this condition (Verner-Jeffreys & Taylor, 2015).

Rainbow trout is Italy's most widely farmed freshwater species, with a total annual production of close to 35.000 tons (FEAP, 2020). Three hundred and sixty farms, mainly concentrated in the north of Italy, account for about 70% of total production. Within the Italian freshwater fish farming context, organic production is a growing sector (ISMEA, 2021; D'Agaro et al., 2022).

This epidemiological study aims to estimate the prevalence of RMS in Italian rainbow trout farms through a questionnaire-based survey. It is an attempt to define more precisely the disease's distribution and impact on a national level in Italy.

## 2. Methods

Data were obtained from a short questionnaire designed to estimate the presence of RMS between 2020-2021 in Italian rainbow trout farms (Figure 1). The questionnaire was based on a version (Schmidt et al., 2018) previously used in the Danish rainbow trout sector.

Three hundred and twenty site managers and field veterinarians (one for each site) were contacted through API (Italian fish farmers association), SIPI (Italian association of fish pathologists), or directly (in person or by phone) during late 2021 and early 2022. Dr Massimo Orioles, University of Udine (Lead author) interviewed the farmers and veterinarians and collected the data.

The questionnaire was composed of 17 questions, including a combination of open (2), closed (9) and multiple-choice (6) questions. The questions were based on general characteristics of the farm (name, type of farm and production, geographical localization, water source) and on specific details about RMS outbreaks, including costs associated with the disease and therapeutic intervention. Details included water temperature, the season when the outbreak was observed, the size of fish affected, the number or percentage of tanks affected, the number or percentage of fish affected within each unit, the type of treatment used, the possible source of infection and potential recurrence of the disease in the same batch of fish. The case definition for the presence of RMS includes the appearance of focal to multifocal chronic hyperemic, usually raised, non-ulcerative lesions between 5 mm to several cm in diameter; affected fish were generally in good condition, with no mortality and show normal behaviour (Oidtmann et al., 2013). Furthermore, the farmers and veterinarians were asked whether the diagnosis was made through laboratory analysis.

## 3. Results

A total of 109 (out of 320 contacted) rainbow trout farms responded to the questionnaire through their farm site health managers and consultant veterinarians. In 8 cases, the farm site health managers were personally guided through the questionnaire; in 11 cases, farm site health managers responded by email; in 90 cases, the answers were provided by email from experienced field veterinarians regularly consulting the farm in the period in question. Out of the 109 responses, 83 were located across the north of Italy. The estimated total production of these 109 farms represented about 64% of the total Italian trout production (approximately 34.400 tons - FAO 2019).

RMS was observed at least once in 31 out of 109 sites during 2020-2021. Farms with reported RMS were distributed across 11 regions (Figure 1).

Figure 1 illustrates the distribution of respondents among regions and the number of farms where RMS has been observed.

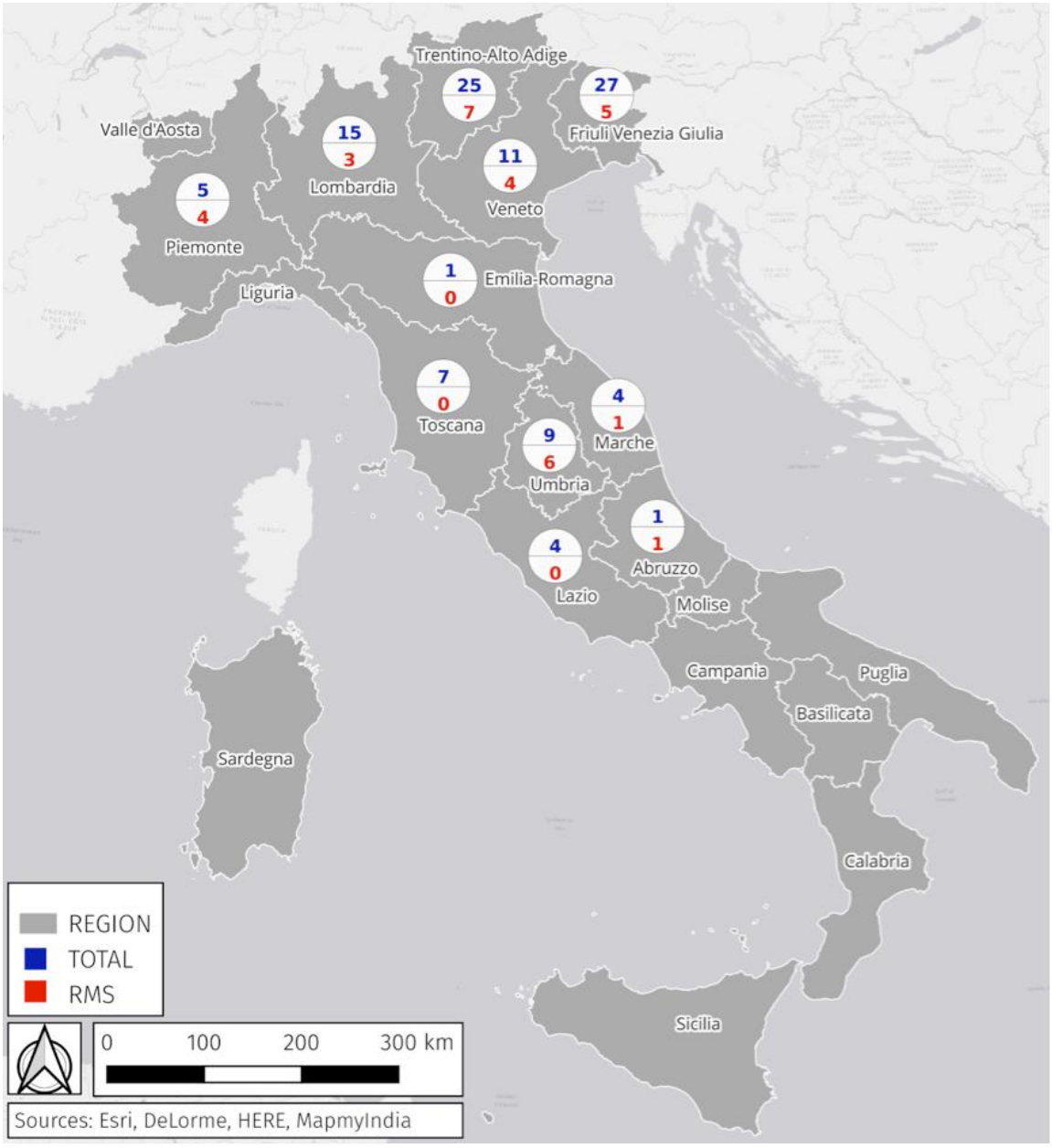



**Figure 2:** Presence of RMS in Italian rainbow trout farms through a questionnaire-based survey. The total number of questionnaires completed and received from farmers and field veterinarians are in blue; the number of sites where RMS has been presumptively noticed is in red.

The 31 sites were all medium and large-sized farms, with a mixed type of production strategy based on both portion size trout product (300-400 grams) and larger fish (up to and more than 500gr). Water was predominantly sourced from rivers (27 out of 31 cases). During outbreaks, the water temperature has been reported to fluctuate between 5° and 15°C. No geographical pattern for positive farms, where water was coming from the same water source or distributed within the same area, was found within each region or river basin area.

Diagnosis in all cases was based only on clinical features and necroscopic findings. No further analysis was performed to confirm the diagnosis. In 12 of 31 (38,7%) cases, the disease was observed more frequently in the last 5 years. Most commonly [14/31 (45,1%)], 10-30% of tanks were affected, while in 10 cases (32,2%) the disease spread through more than 50% of the tanks.

In most cases (20/31 (64,5%), relatively few fish in a unit (up to 10%) showed typical clinical signs of RMS, while the presence of clinical signs in 10-50% of the population in a unit was reported in 11 (35,5%) cases. Most commonly, affected fish were close to commercial portion size (250-500g; 41,9% of cases) or somewhat smaller (100-250g; 29,0% of cases). In 7 cases (22,6%), RMS was observed in trout of less than 100g (all reported by the same veterinarian in the same region). RMS in trout >500g was only reported from two farms (9,67%).

Twenty respondents (64,5%) reported that recurrence in the same batch of fish was observed; in 22 cases (70,9%), the infection was believed to be transmitted through the water from upstream farms and in 8 cases (25,8%) from the introduction of fish or eggs. RMS lesions were most commonly observed in spring, and active RMS lesions were never observed during summer.

The farmers opted not to treat the disease in 14/31 (45,1%) cases, all of which were in cases where 0-10% of tanks were affected. Oxytetracycline was used in 14 cases (45,1%), with the majority used in most severe outbreaks, where more than 50% of units were affected with 10- 50% of the population involved. In 3 cases (9,6%) treatment was given, but details were not specified.

RMS-associated costs were not known or determined in 10 cases (32,2%). In 10 cases, the respondents considered RMS-associated costs mainly related to therapies. Eight respondents out of 31 (25,8%) stated that this disease has no associated costs, while only in 2 cases the commercial downgrade of carcasses was claimed as the only cost. In both cases, RMS was observed in over 50% of units affecting 10-50% of individual fish of all sizes.

No concerns over feed conversion, mortality or growth rate were raised. The percentage of RMS-associated production costs was never specified. Details of the results are summarized in Table 1.

**Table 1:** Summary of the main results of the questionnaire-based survey on RMS presence in Italian farms during 2020-2021. One hundred and nine farms responded and 31 were RMS positive.

| **QUESTION** | **TOTAL (out of 31)** |
|---|---|
| | |
| **Has RMS been more frequently seen in the last 5 years?** | |
| No | 14 |
| Yes | 12 |
| Not determined | 5 |
| **Season of outbreak(s)** | |
| Spring | 27 |
| Winter | 17 |
| Autumn | 15 |
| Summer | 0 |
| **Number of units** | |
| 0-10% | 6 |
| 10-30% | 14 |
| >50% | 10 |
| 100% | 1 |
| **Percentage of fish affected inside the unit** | |
| 0-10% | 20 |
| 10-50% | 11 |
| **>50%** | 0 |
| **Size of affected fish** | |
| <100g | 7 |
| 100-250g | 9 |
| 250-500g | 13 |
| >500g | 2 |

| **Perceived route of transmission** | **TOTAL** |
|---|---|
| Water | 23 |
| Eggs | 4 |
| Movement of fish | 4 |
| **Recurrence of infection** | **TOTAL** |
| Yes | 20 |
| No | 6 |
| Not determined | 5 |
| **Therapies used** | **TOTAL** |
| No | 14 |
| Yes, antibiotic (Oxytetracycline) | 14 |
| Yes,unspecified medicated feed | 3 |
| **Costs associated with RMS** | **TOTAL** |
| Not determined | 10 |
| Therapies | 10 |
| None | 8 |
| Unspecified | 1 |
| Commercial downgrading | 2 |

## 3. Discussion

As reported by the results of the 25th Annual Workshop of the European Reference Laboratories for Fish Diseases (DTU-AQUA, Denmark, https://www.eurl-fish-crustacean.eu/fish/annual-workshop/26th-aw-2022/25th-aw-presentations), RMS remains to date a significant concern in rainbow trout farming, even though the disease is generally considered manageable by health managers and veterinarians. This is the first RMS survey conducted in Italy to estimate prevalence and distribution on the Italian territory. The survey was considered acceptable to represent the Italian sector as the responding farms, mainly concentrated in the north of Italy, were estimated to be around 64% (about 22.000 tons) of the total national rainbow trout production. We considered the content validity of the answers good, as the survey was already pilot tested in Denmark (Schmidt et al., 2018), and the questions were concise and easy to understand, realistically covering all the information needed and obtainable from farmers and field veterinarians. Although no diagnostic confirmation through histology nor molecular biology techniques was performed, thereby introducing a potential bias in the evaluation of the prevalence, RMS presumptive diagnosis

was made mainly by experienced field veterinarians (the actual respondents in 82% of the cases). Despite this, the accuracy of these data remains essentially unknown, and most of the answers could be prone to recall bias as they are not based on a specific recording system. Data described here should be interpreted with this in mind, and the use of data from computer-based management systems integrated for farm use could solve this issue.

Survey responses indicate that RMS observed in Italian farms has similar features to what is generally described in the literature (Metselaar et al., 2022): outbreaks occur more often in springtime with water temperatures around 8-10° C and up to 15-16°C, with no substantial difference in water temperature between negative and positive farms. Contrary to what was reported in Danish farms (Schmidt et al., 2018), where the disease outbreaks are seen all year round, RMS in Italy is not seen during summertime, as expected, due to high water temperature.

RMS outbreak most commonly affects 10-30% of units (45,1%) and up to 10% of the fish in these units (61,2%). Nevertheless, it is worth considering that these percentages may be underestimated, as small lesions can often pass unnoticed since RMS does not directly impact the growth rate, mortality, or feed conversion ratio. In addition, no geographical pattern for positive farms was found within each region or river basin area, where water was coming from the same water source or distributed within the same area.

RMS in Italian farms seems to follow a similar kinetic within the farms as reported in previous studies (Verner-Jeffreys et al., 2006). Once it is observed on a farm, fish will develop more severe clinical signs of RMS in approximately 3 to 4 weeks and resolve in a further 8 to 12 weeks. It is a rolling infection, as during this period of about 3 to 4 months, some fish will start healing, whereas others will start showing signs with a long latency.

Oxytetracycline is the most common and effective antimicrobial used in the cases reported here. This is reported in early literature (Verner-Jeffreys et al., 2008) and confirmed in experimental studies (Schmidt et al., 2021), but it is no longer favoured due to the long withdrawal period and the

treatment-related costs (Verner-Jeffreys et al., 2015). Interestingly, antibiotic use reported in previous questionnaire-based studies (Schmidt et al., 2018) is rare and observed as poorly effective.

It is a common practice to retain fish with RMS, as the condition spontaneously resolves, and there is no reported impact on either survival or growth. This disease management practice is more suitable for producers rearing large trout for the table or recreative fisheries, as opposed to portion table trout producers or organic farmers.

Furthermore, the survey shows that antibiotics are most commonly used when the disease affects more than 50% of the tanks, reflecting the relatively benign nature of the disease and the fact that treatment is used only when morbidity appears relevant.

Unusual findings in this study include that rainbow trout of less than 100g were relatively often affected and that RMS is reported to recur in the same batch of fish. Only three recent descriptions of juvenile trout affected by RMS are available in the literature (Oh et al., 2019; Orioles et al., 2022; Schmidt et al., 2022); otherwise, the disease is reported to affect fish of more than 100g only.

Recurrence of clinical signs in the same fish is generally not considered possible, or at least very rare (Schmidt et al., 2018), as it seems exposure of fish to RMS can trigger the development of natural immunity (Metselaar et al., 2022; Schmidt et al., 2022). Also, it is difficult to follow the same batch of fish across all production cycles. This should be further investigated in future studies.

Regarding possible transmission routes, from the results of our study, it is evident that veterinarians and farmers think water is the primary source of infection, while the movement of eggs and fish within and across the farms is considered to have a lower potential impact on the transmission of the condition. Even though early studies on the subject reported that sites with very few suppliers of live fish and ova have a lower probability of infection due to their limited contact with other sites, we could not specifically investigate this risk factor here.

No other freshwater species were observed having RMS-like disease. It is worth noting that brown trout were also recently confirmed to be a susceptible species (Schmidt, 2019; Scott, 2022).

When considering biosecurity measures and predisposing factors for RMS outbreaks, no definitive conclusions can be drawn from the data gathered from this survey, but few speculations are possible. Compared with previous surveys (Adam, 2010; Schmidt et al., 2018), in our study, the number of fish deliveries to a farm or connections through the river network may not have any impact on the farms' chances of developing the disease. Negative and positive farms were often both supplied by the same water source, and none of the positive farms obtained fry from more than 4 different suppliers. This has been recognized as a risk factor (Adam, 2010). In addition, none of the positive farms was based on closed systems or operated solely manual handling of fish; all positive farms and about 90% of negative farms used mechanical methods of handling fish. On the other hand, all small-sized, closed farms that use mainly manual handling and hand netting were negative. As previously reported (Adam, 2010), avoiding mechanical handling may represent a protective factor.

Stocks held on the same water supply were reported to develop the same conditions in positive farms. This is compatible with what is reported in previous studies (Verner-Jeffreys et al., 2008).

Currently, there are no specific biosecurity measures for RMS, mainly due to the need for more knowledge about the biology of MLOs. Generally speaking, a closed farm where no fish are imported and movements of fish are restricted will be less likely to contract new diseases. This only sometimes seems to be the case for RMS, as described in recent reports within an isolated RAS (Orioles et al., 2022c). Horizontal transmission by direct or indirect contact is supported by cohabitation studies (Verner-Jeffreys et al., 2008; Jorgensen et al., 2019).

Vertical transmission could be possible as it was suggested that batches of eggs initially introduced the disease (Metselaar et al., 2022). Since the putative causative agent is likely an obligate intracellular bacterium, this could also indicate a higher risk of vertical disease transmission. It was not possible to investigate the role of egg transfer inside or across different sites due to the lack of respondents' collaboration and compliance. Eggs from the same supplier were used both in negative

and positive farms, so the link was considered only circumstantial, as reported in the literature (Verner-Jeffreys et al., 2008).

The role of vertical transmission needs to be investigated further, and the importance of this transmission route is discussed in more detail with health managers and veterinarians. Another challenge is the presence of wild stock swimming freely in the waterbody.

Finally, no apparent connection between the type and intensity of feeding and RMS was found.

In conclusion, RMS is still present in Italian rainbow trout farming, affecting about 30% of farms. Despite being widespread, the disease is not perceived as a major concern in the Italian trout farming industry, mainly due to its intermittent nature, low mortality and spontaneous resolution. However, after 20 years since the first observation in Europe (and even earlier considering Fleury et al., 1985), this survey reiterates some knowledge gaps about the disease, such as the biology, distribution, genomics, transmission routes, host specificity and pathogenicity of MLO. This has implications for biosecurity and management measures, as well as the impact of MLO in wild populations. Furthermore, we still need a reasonable estimate of the economic impact of RMS on the trout sector.

# 3. Observations from first report of outbreaks in Bosnia and Herzegovina

The present contribution describes the first occurrence of RMS outbreaks in Bosnia and Herzegovina commercial trout farms. Bosnia and Herzegovina freshwater aquaculture sector consists of approximately 100 small-sized farms and 5–6 larger ones (author personal communication). The total annual production varies in between 3,000 and 4,000 tons, and rainbow trout is the most important farmed species (FAO, 2020). Traditionally, up to now the small-size farms have used primarily their own broodstocks, but this tendency has decreased in recent years. Currently, many of them purchase fingerlings from larger farms or small hatcheries in Bosnia and Herzegovina. Conversely, all the specialized large-scale producers buy the eggs from USA, Spain or Italy. The water provision for these aquaculture plants derives mostly from local rivers. The present outbreaks occurred in two large intensive rainbow trout farms (here referred as farm A and farm B), during the late winter and spring of 2020. In farm A, fish were reared up to commercial size in concrete raceways supplied by river water (T 8,5° in winter–9,5°C in summer) deriving from nearby snowfields and fed with a commercial pelleted diet. Rearing density was about 40–50 Kg/m3. The affected individuals, at a percentage of 20%–25%, were kept in several basins. Their size was approximately 250–300 g. In farm B, located in a geographical area quite distant from the previous one, fish were similarly reared up to commercial size in concrete raceways supplied by river water (T 8° in winter–11°C in summer) and fed with a commercial pelleted diet. Rearing density was about 25 Kg/m3. The affected fish (30%–35%) were distributed in a limited number of basins within the whole farm. Their size was approximately 300 g. Mortality was absent in both farms. Recorded individuals showed signs of disease ascribable to RMS, according to the criteria proposed by Oidtmann et al. (2013). Concerning the farm A, the disease already occurred in the

previous years with peaks in prevalence, sometimes as high as 50%. In the farm B, RMS already occurred in a sporadic but not impactful form. Currently, the disease appeared with a more severe course in both farms. Regarding the sanitary situation, both surveyed farms are free from SEV and IHN, as reported by the Croatian Veterinary Institute (Lab for Fish and Molluscs diseases). Sleeping disease and furunculosis are observed instead, whereas ERM (caused by biotype 1 and 2 Yersinia ruckeri) is recorded every year. Seventeen symptomatic fish were sampled in Farm A, and eighteen in Farm B, then killed with an overdose of MS222 (500 mg/L). All individuals were submitted to necropsy, and digital images were captured in order to document the macroscopic lesions. For the histological examination, tissue samples obtained from skin were fixed in 4% neutral buffered formaldehyde and then processed by an automatic histoprocessor (TISBE, Diapath, Italy) to be embedded in paraffin (ParaplastPlus, Diapath). Serial 5-µm sections were stained with haematoxylin–eosin (HE). Three hyperplastic spleens collected from farm B trout were used to prepare smears and impressions, and stained with Giemsa and Macchiavello stain as previously described (Culling, 1974). Slides were observed under optical microscope, and relevant digital histological images were captured. The DNA extraction and purification was carried out in the same seventeen symptomatic fish sampled in Farm A and eighteen symptomatic fish sampled in Farm B. Five healthy fish as control group were collected from a commercial rainbow trout farm, which is a RMS-free facility, in order to be used as reference samples. The tissues (skin/muscle, spleen and head kidney) were hygienically dissected and cut to $\leq 0.5$ cm in any single dimension, before the immersion in ethanol for DNA extraction. All samples including infected and controls underwent DNA extraction using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions for animal tissue, and eluted DNA was stored at − 20°C. Samples were lysed (3 hr), before washing andelution were completed. Total genomic DNA was assessed for yield and quality using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). The specific MLO DNA was amplified using PCR according to Galeotti, Manzano, et al. (2017) with few modifications. PCR amplification of the MLO DNA using the primer was carried out in a final volume of 50 µl. First,

PCR was amplified using RLO1/RLO2 (Lloyd et al., 2008) primer in a Bio-Rad thermo cycler (Bio-Rad Laboratories Inc., CA, USA, http://www.bio-rad.com/) with a reaction mixture containing 100 ng DNA, 100 ng of each primer and 1.25 units of Taq DNA polymerase (Thermo Fisher scientific, USA, https://www.therm ofish er.com ) under the following conditions: the amplification protocol: the time of denaturation at 95°C was increased from 2 to 5 min, 35 cycles of 95°C for 30 s, and the annealing temperature was increased from 57°C to 69°C for 30 s, 72°C for 30 s instead of 60 s and a final extension at 72°C for 10 min. For each PCR, water has been used as a blank (negative control) to verify that no contaminations were present during the amplification step. Second, nested PCR assay was performed using a primer pair, RiFCfw 5'-AAGGCAACGATCTTTAGTTGG-3' and RiFCrev 5'- CCGTCATTATCTT CCCCACT-3', within the Amplicon obtained by the first step using the primers RLO1 and RLO2. The amplification was obtained using 2 µl of the first step as template following the protocol: 95°C denaturation for 5 min, 35 cycles of 95°C for 45 s, 54°C for 45 s, 72°C for 45 s and a final extension at 72°C for 7 min.

Subsequently, 5 µl of the PCR product was analysed by 2% agarose gel electrophoresis stained with ethidium bromide and visualized with an UV transilluminator. PCR product molecular weight was determined using a 50-bp DNA ladder (Thermo Fisher Scientific, Pittsburgh, PA, USA). To identify the type MLO isolate used in the present study, PCR products were then purified using a QIAquick Purification kit (Qiagen, https://www.qiagen.com/it/) and directly sequenced using the RiFCfw 50/RiFCrev AO18SF/R primer set (Eurofins Genomics). Using a BLAST search, the sequences obtained were compared with those published in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

In fish deriving from both farms, gross skin lesions, single or multiple, consisted in small to large areas, ranging from 1x1 to 3x4 cm, flat or protruding, round/oval shaped, pink or pale red, sometimes displaying scattered petechial haemorrhages, sometimes with a desquamation in the central part of the lesion, often covered by serous/ fibrin exudate (Figures 1 and 2). In the farm A, these lesions were single, and flank located in 7 out of 17 individuals, and multiple in 10 out of 17

individuals. In the farm B, the lesions were single, and flank located in 7 out of 18 individuals, and multiple in 11 out of 18 individuals. Spleens showed increased volume and rough surface in 2 out of 17 individuals in farm A, instead 8 out of 18 individuals in farm B. Once cut, the organ showed congestion. No lesions were detectable in the other organs. Histology revealed a severe skin inflammatory disease affecting all the skin layers sometimes extending to the underlying muscular tissues (Figures 3 and 4). The inflammatory infiltrate was composed by numerous lymphocytes and macrophages mainly localized within the stratum compactum and spongiosum. In the latter, the scale pockets were often cancelled or obscured by the inflammatory cells and numerous haemorrhages were also present.

**Figure 1:** RMS gross skin lesions in symptomatic rainbow trout of farm A. Multifocal to coalescing round shaped, raised and pale pink-red lesions.

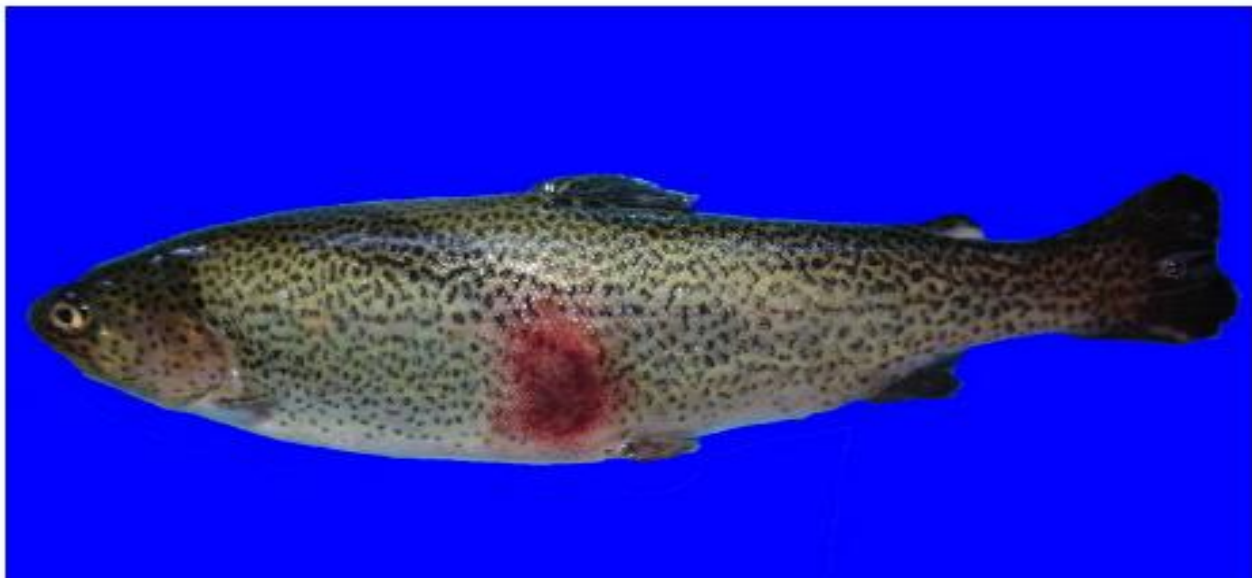


**Figure 2:** RMS gross lesion in symptomatic rainbow trout of farm B characterized by large, locally extensive, oval-shaped and raised haemorrhagic area.

The epidermis appeared variably eroded and/or infiltrated by inflammatory cells, whereas the hypodermis and, in some cases, the deep muscular tissue appeared heavily infiltrated by the same inflammatory cells. Cytological examination of spleen smears revealed a mixed population of cells, including monocytes containing numerous intracytoplasmic oval to round, 0.5–1.5 μm diameter, microorganisms that stained red with Macchiavello's method (Figure 5). The three spleen specimens submitted to this stain resulted also positive by PCR for MLO.

The amplification of 188 bp (RMS/MLO) after nested PCR was considered as positive in this study. From the Farm A, skin/ muscle analysis indicated 11 positive out of 17 symptomatic trout (64.70%). Whereas from Farm B, 16 out of 18 individuals were positive (88.88%). No amplification of MLO DNA was recorded in skin/ muscle samples from the control RMS-free farm. Five fish out of 11 positive from Farm A resulted positive in all the investigated tissues: skin/muscle, spleen and head kidney. In Farm B out of 16 positives, 11 indicated amplification in all the tissues analysed.

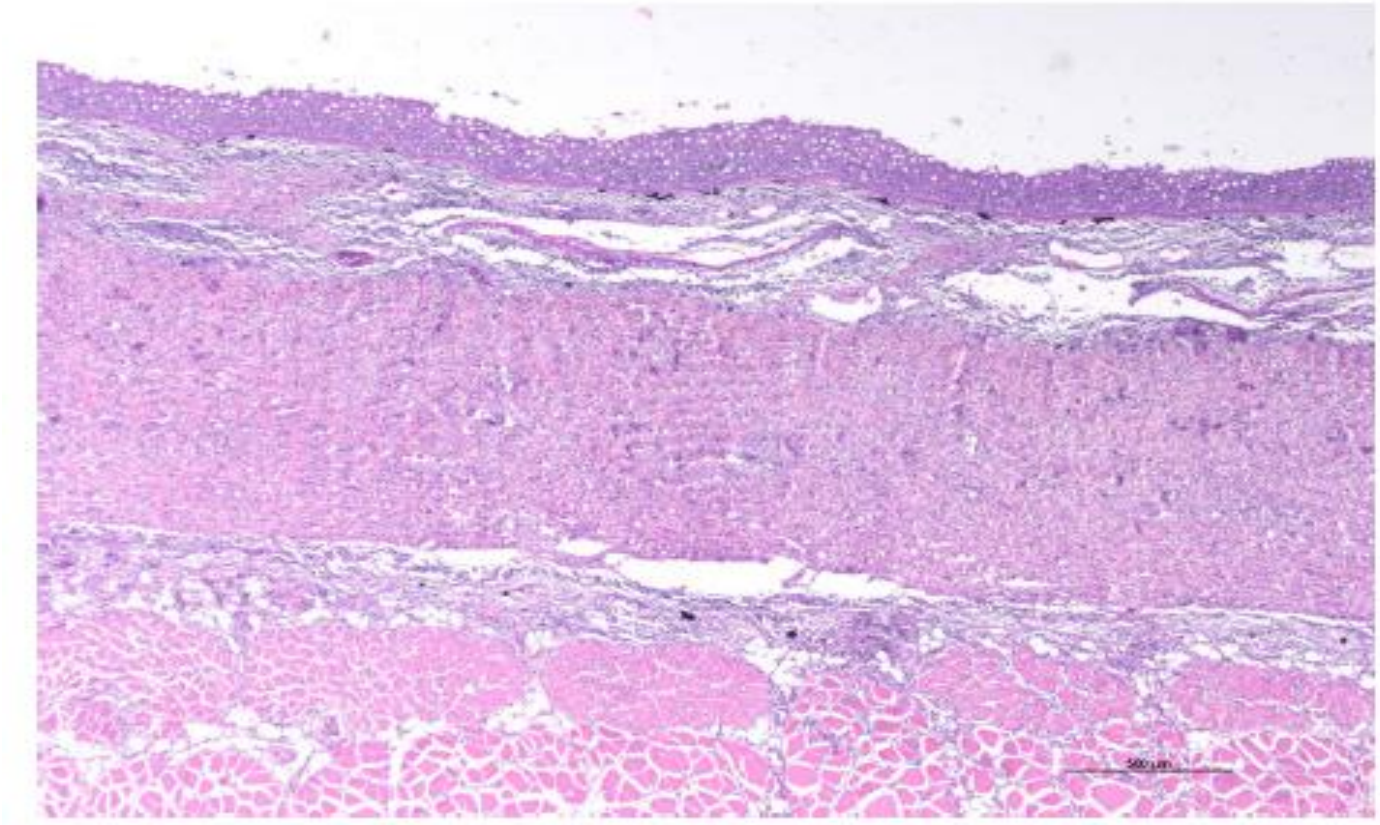


**Figure 3:** RMS microscopic skin lesions in symptomatic rainbow trout of farm A. Histology reveals inflammation in all skin layers and the underlying muscular tissue. The epidermis is thinned, and the scale pockets appear severely infiltrated by lymphocytes and monocytes/macrophages with complete scale resorption. The stratum compactum of the derma and the hypodermis are thickened by the presence of inflammatory infiltrate (H&E).

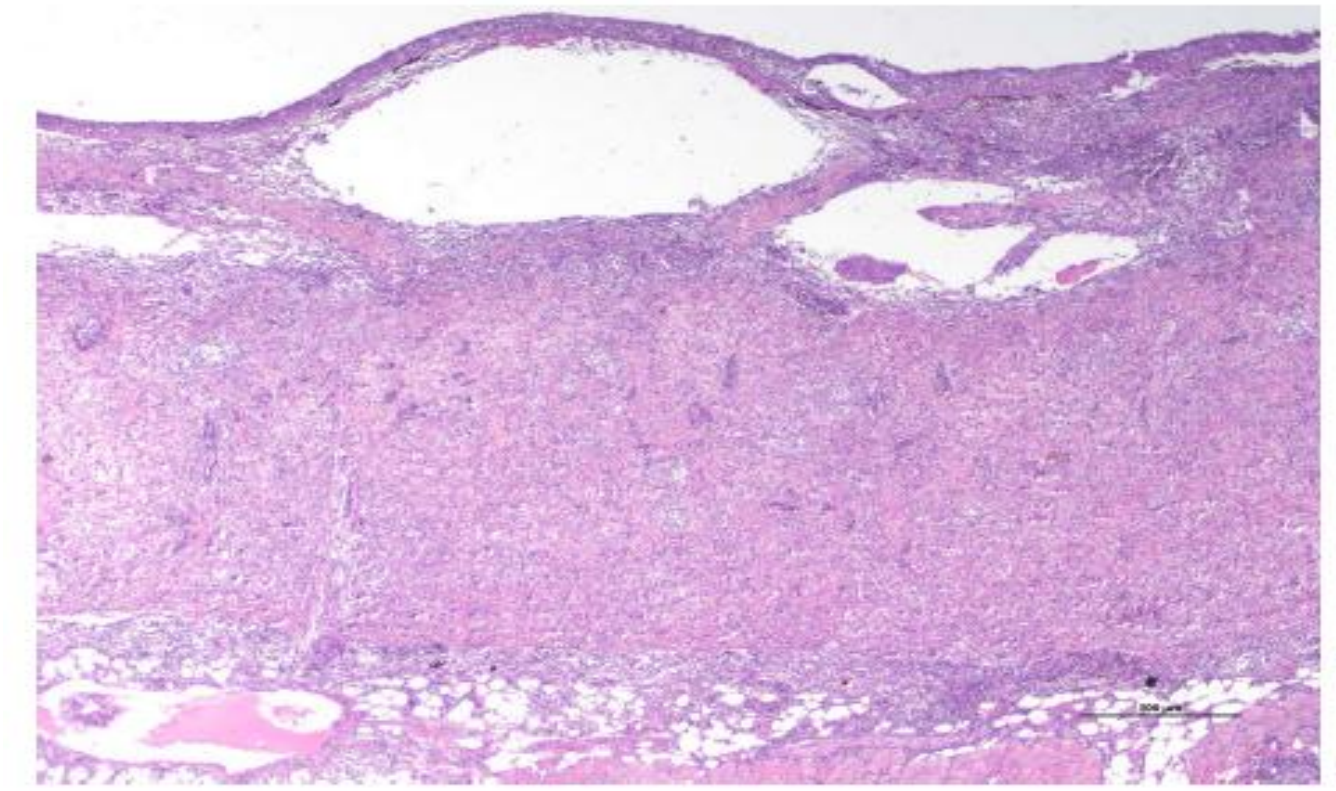


**Figure 4:** RMS microscopic skin lesions in symptomatic rainbow trout of farm B. Histopathological examination reveals inflammation extending from dermis to the deep muscular tissue. The stratum spongiosum of the derma appears severely oedematous and expanded by lymphocytes and monocytes infiltrating scales pockets. The scales are still present. The stratum compactum and the hypodermis are thickened and heavily infiltrated by inflammatory cells (H&E).

According to the standardized method proposed by Oidtmann et al. (2013) for the description of rainbow trout skin disorders of uncertain aetiology, it is possible to refer both episodes to RMS. It has been reported in the literature that RMS could be transmitted through water and contaminated eggs (Verner-Jeffreys et al., 2008). Given that the farm A water supply comes via river from nearby snowfields, it is unlikely that RMS could have spread through the aquatic route. On the other hand, the farm B water supply comes from river water, which is connected with other trout farms; in this case, a water-based transmission might be possible. This river is tributary of the Sava river stretch entering Croatia. Moreover, both farms purchase variable percentages of eggs from external sources (personal communication of farm managers), specifically farm A for about 50%, whereas farm B

for 100%. The authors hypothesize that the disease could have spread through contaminated eggs, especially in the case of farm A for the reason mentioned above. Contaminated eggs represent a further risk factor of infection in the case of farm B, in association with the possible entrance of the pathogen by the river water eventually contaminated. RMS has been sporadically but unofficially reported for many years in Bosnia and Herzegovina. Trout farming production in this country has gradually increased in the last 15 years and RMS seems to be more frequently reported in the last five years, since the activity of many intensive trout farms has started. The spreading of RMS from Bosnia and Herzegovina to other nearby countries like Croatia is certainly possible, considering the presence of many transboundary waters.

Considering the cases reported in this article in Bosnia and Herzegovina and the recent descriptions of RMS in Slovenia (Galeotti, Ronza, et al., 2017) and Denmark (Schmidt et al., 2018), the authors think RMS will be spreading quickly throughout all Europe. The relative Bosnia's geographical, orographical and economical isolation makes the infection through egg's contamination likely, even though no further speculations are possible due to the uncertain aetiology and pathophysiology of RMS, and to the absence of

the literature evidences on MLO/RLO molecular detection in trout eggs. Therefore, in the future a sanitary control of the eggs entering the farms by means of molecular analysis for MLO would be useful.

# 4. Red mark syndrome of trout (oncorhynchus mykiss; Walbaum, 1792): histopathological scoring and correlation with gross lesions

## 1. Introduction

Early clinical signs are characterized by pale yellow/grey flat patches with central reddening, mainly on the fish flank, or opaque raised patches, with increased mucus production (Schmidt-Posthaus et al., 2009; Verner-Jeffreys et al., 2008). The lesion evolution includes an increase in size, elevation and redness of the wounds. The diameter of the lesions ranges from 5 mm to several centimetres;they appear round or ovoid, elongated, with progressive scale loss. Ulceration is not a common finding (Ferguson et al., 2006; Noguera, 2008; Verner-Jeffreys et al., 2006, 2008). The histology of the affected skin reveals a mild-to-severe dermatitis, with intact epidermis and massive infiltration of inflammatory cells, such as lymphocytes, plasma cells and macrophages (involving the stratum spongiosum [SS], the scale pockets [SP] and the stratum compactum [SC]). A severe infiltration of the same cells is consistently observed in the hypodermis and underlying muscle (Galeotti, Manzano, et al., 2017; Galeotti, Ronza, et al., 2017; McCarthy et al., 2013; Schmidt-Posthaus et al., 2009; Verner-Jeffreys et al., 2008). Most of the investigations described in the literature have aimed to understand the aetiopathogenesis of the disease, whereas relatively few papers have focused on a detailedanalysis of its histological features in order to both define a classification of the lesions during their evolution and further understand the pathogenesis of this disease, which is still far from being clear. The present work aimed to describe the development of the lesion from the histological point of view, starting from the early

stages to the fully advanced lesions, and to correlate the scoring of skin histological patterns with selected macroscopic features of the lesions.

## 2. Materials and methods

### 2.1 Case study and sampling

The disease was identified and surveyed in five rainbow trout farms (namely A, B, C, D and E) located in Northern Italy, throughout the spring–autumn period from 2011 to 2015, when the water temperature was between 9 and 10°C. The percentage of affected fish in the farms was 10%–15%, and their mean weight was around 500 g. Fish did not show alteration of behaviour or other signs of diseaseexcept for the cutaneous gross changes ascribable to RMS; mortalitywas absent. Forty-six symptomatic fish were sampled and killed by an overdose of MS-222 (300 mg/L); the number of individuals collected from each farm was as follows: A = 10, B = 10, C = 10, D = 8 and E = 8. Samples of skin, heart, spleen, kidney, liver, gill, brain and gut were included for the histological analysis. A single skin lesion was collected from each symptomatic fish, and then subjected to gross classification and processed for histology. For those individuals presenting multiple skin lesions, the most severe were selected as representative for this purpose.

### 2.2 Gross classification criteria for RMS skin lesions

To classify the skin lesions of RMS-affected rainbow trout on the basis of gross appearance, the authors employed grading criteria as described by Galeotti et al. (2013) with some modifications. The included criteria for descriptive methodology were chosen considering a high number of skin lesions observed throughout the last years in Italian, Slovenian and Bosnian rainbow trout farms with full-blown RMS episodes. The five macroscopic parameters employed in this study to grade RMS skin lesions were as follows: size, colour variation, exudate, visible scales and erosion. Size

ranging from few mm to several centimetres in diameter and considering the major diameter for the oval or elongated lesions. Colour variation from pale grey-whitish to bright red haemorrhagic patches. Presence of exudate: mild (sharp demarcated pale areas with slightly raised scales), moderate (flat or slightly raised whitish patches, covered with clear serous exudate associated with multifocal petechiae) or severe (flat or slightly to moderately raised, shiny patches covered with whitish to yellowish serous fibrinous exudate associated with haemorrhages). Presence or absence of visible scales within lesion. Presence of erosion within lesion defined as greyish, sometimes slightly depressed area, usually centrally located and surrounded by prominent redness. According to these macroscopic descriptors, the lesions were classified into three categories: type I (mild), type II (moderate) and type III (severe), as shown in Table 1. For the inclusion within a category, the lesion evaluated must possess at least three parameters, as described in Table 1. Sampled trout sometimes showed concomitant multiple lesions, sometimes confluent or belonging to different categories. In this study, from each trout under investigation, only one lesion was examined considering the most severe in order to correlate it with the histological features.

**Table 1:** Classification of RMS skin lesion based on the macroscopic parameters.

| Parameters | Type I | Type II | Type III |
|---|---|---|---|
| Size | Up to 1 cm | 1–2 cm | Above 2 cm |
| Colour | Pale grey, whitish, slight redness | From grey to reddish with petechiae | Marked redness and haemorrhages |
| Exudate | Mild | Moderate | Moderate or severe |
| Scale | Usually visible | Mild scale loss | Evident scale loss |
| Erosion | Absent | Absent or rare | Frequent |

### 2.3 Histology

Tissue samples obtained from skin/muscle, brain, gill, liver, heart, spleen, kidney and gut were fixed in 4% neutral buffered formaldehyde at 4°C overnight. After fixation, samples were

equilibrated at room temperature, processed by an automatic histoprocessor (TISBE tissue processor, Diapath) and embedded in paraffin (Paraplast Plus, Diapath). Serial 5-µm sections were obtained by a programmable microtome (Reichert-Jung 2050) and then stained with haematoxylin and eosin and Van Gieson Weigert trichrome. The specimens were examined with a light microscope (Leica DMRB), and images were acquired with a Nikon system. A single observer (MG) scored the histopathological parameters on skin samples, using semi-quantitative criteria. For each parameter, the score ranged from 1 to 4, as follows: 1 = absence of the lesion, 2 = mild lesion, 3 = moderate lesion and 4 = severe lesion. The parameters evaluated are shown in Table 2.

**Table 2:** median values and range for each group of the scored histological variables and results of the Spearman correlation test with the three gross types for each variable.

| Site | Histological variable | Gross skin lesion | | | *R* Spearman's test* |
|---|---|---|---|---|---|
| | | Type I | Type II | Type III | |
| Epidermis | Absence | 1(1–2) | 2(1–4) | 3(1–4) | 0.64* |
| | Exocytosis | 1(1–2) | 2(1–4) | 3(2–3) | 0.47* |
| | Necrosis | 1 | 2(1–4) | 2(1–4) | 0.45* |
| Dermo-epidermal junction | Lymphocyte infiltration | 2(1–3) | 3(3–4) | 4(3–4) | 0.53* |
| | Lichenoid-like detachment | 1 | 1(1–3) | 1(1–3) | 0.12 |
| | Necrosis | 1 | 3(1–4) | 3(1–4) | 0.40* |
| | Haemorrhage | 1 | 2(1–3) | 2(1–4) | 0.41* |
| Dermis—stratum spongiosum | Cellular infiltration | 2(2–3) | 3(3–4) | 4(3–4) | 0.48* |
| | Vessel congestion | 1 | 3(2–4) | 4(3–4) | 0.59* |
| | Haemorrhage | 1 | 3(1–4) | 3(1–4) | 0.37* |
| | Oedema | 1 | 2(1–4) | 2(1–4) | 0.24 |
| | Necrosis | 1(1–2) | 3(1–4) | 4(1–4) | 0.44* |
| | Thrombosis | 1 | 1(1–4) | 2(1–4) | 0.34* |
| Dermis—scale pockets | Absence of scale pockets | 1(1–2) | 1(1–3) | 3(1–4) | 0.65* |
| | Pocket inflammatory infiltrate | 1(1–2) | 3(2–4) | 4(1–4) | 0.47* |
| | Pockets with oedema/fibrin | 1(1–4) | 2(1–4) | 2(1–4) | 0.23 |
| | Scales in re-absorption phase | 3(1–3) | 3(1–4) | 1(1–4) | −0.43* |
| | Inflammatory infiltrate below scales | 1(1–2) | 2(1–4) | 1(1–4) | −0.27 |
| | Absence of scales | 1 | 3(1–4) | 4(2–4) | 0.84* |
| | Scale regeneration | 1 | 1 | 1(1–4) | 0.33* |
| | Multinucleated cells | 1 | 1 | 1(1–4) | 0.41* |
| Dermis—stratum compactum | Cellular infiltration | 2(1–3) | 3(2–4) | 4(3–4) | 0.66* |
| | Vessel congestion | 1 | 2(1–4) | 3(1–4) | 0.45* |
| | Haemorrhage | 1 | 2(1–4) | 2(1–4) | 0.31* |
| | Oedema | 1 | 1(1–4) | 1(1–3) | 0.20 |
| | Thickening | 1(1–2) | 3(1–4) | 4(2–4) | 0.58* |
| | Loss of structure | 1(1–3) | 3(1–4) | 4(2–4) | 0.67* |
| Hypodermis (subcutaneous tissue) | Cellular infiltration | 3(1–3) | 3(2–4) | 4(2–4) | 0.48* |
| | Vessel congestion | 1 | 2(1–4) | 2(1–4) | 0.27 |
| | Haemorrhage | 1 | 2(1–4) | 1(1–4) | 0.10 |
| | Enlarged lymphoid vessels | 1 | 1(1–3) | 1(1–4) | 0.26 |
| | Granulomatous reaction surrounding vessels and nervous fibres | 1 | 2(1–3) | 4(1–4) | 0.67* |
| | Multinucleated cells | 1 | 1 | 1(1–4) | 0.37* |
| Muscle | Lymphocyte infiltration | 1(1–3) | 3(2–4) | 4(2–4) | 0.62* |
| | Granulomatous reaction surrounding the myofibres | 1 | 2(1–4) | 3(1–4) | 0.56* |
| | Miosepta infiltration | 1(1–3) | 2(1–4) | 3(1–4) | 0.49* |
| Peritoneal serosa | Lymphocyte infiltration | 1(1–2) | 2(1–4) | 2(1–2) | 0.35 |
| | Visceral adhesions | 1 | 1(1–4) | 1 | 0.04 |

*Note:* For each parameter, the score ranged from 1 to 4, as follows: 1 = absence of the lesion; 2 = mild lesion; 3 = moderate lesion; and 4 = severe lesion. Legend: median (min–max).

*$p < .05$.

## 2.4 Proliferating cell nuclear antigen (PCNA) immunohistochemistry

Twenty skin/muscle samples, representative of the scored lesions (6 macroscopically scored as type I, 5 as type II and 9 as type III), were evaluated with immunohistochemistry using an anti-PCNA antibody

to assess the cell proliferation rate within the lesion. Four-micron-thick formalin-fixed, paraffin-embedded sections were deparaffinized for 30 min in Solvent Plus (Carlo Erba) and then hydrated in a series of graded alcohols. Subsequently, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water for 30 min at room temperature; sections were then treated in citrate buffer (pH 6.0) for 10 min in a microwave oven (750 W) for antigen retrieval. Non-specific binding was blocked by incubating the sections with 5% normal goat serum and 1% bovine serum albumin (BSA) and phosphate-buffered saline (PBS) (blocking solution) for 1 hr at room temperature, and then, sections were incubated overnight at 4°C with mouse monoclonal anti-PCNA (Clone PC10, Cell Signaling Technology) diluted 1:4,000 in blocking solution. A horseradish peroxidase (HRP) polymer conjugate SuperPicture™ Detection Kit (Zymed, Invitrogen) was applied to the sections for 10 min at room temperature to visualize antibody–antigen binding. The immunohistochemical reaction was developed with 3,3-diaminobenzidine solution (Sigma-Aldrich). The slides were counterstained with Papanicolaou haematoxylin, dehydrated in a series of graded alcohols and cleared in xylene before mounting.

## 2.5 Semi-quantitative evaluation of PCNA- positive cells

For each stained section, the percentage of PCNA-positive cells was evaluated (RS, LM) in the epidermis (epithelial cells), the dermis (lymphocytes, endothelial cells and fibroblasts) and the hypodermis (lymphocytes, endothelial cells and fibroblasts) considering four score levels: 0

(negative), 1 (positivity range: 1%–25%), 2 (positivity range: 25%–50%), 3 (positivity range: 50%–75%) and 4 (positivity range: 75%–100%).

### 2.6 Statistical analysis

Score data of skin histological variables, listed in Table 1, and score of PCNA positivity were tested for normality using the Shapiro–Wilk W test. Because the data distribution was not normal, the correla tion of the scored variables with the three types of gross lesions was performed using the non-parametric Spearman rank-order correlation test (CSS Statistica, Statsoft). A p value <.05 was considered significant.

## 3. Results

### 3.1 Gross skin lesions

Lesions were allocated to one of three categories as follows: type I = 7 lesions (Figure 1a, b), type II = 19 lesions (Figure 1c, d) and type III = 20 lesions (Figure 1e, f). The macroscopic examination of visceral organs did not reveal lesions apart from a slightly increased spleen volume in 18 out of 46 (39%) sampled trout. These spleens also showed a rough surface, and upon dissection, they showed congestion.

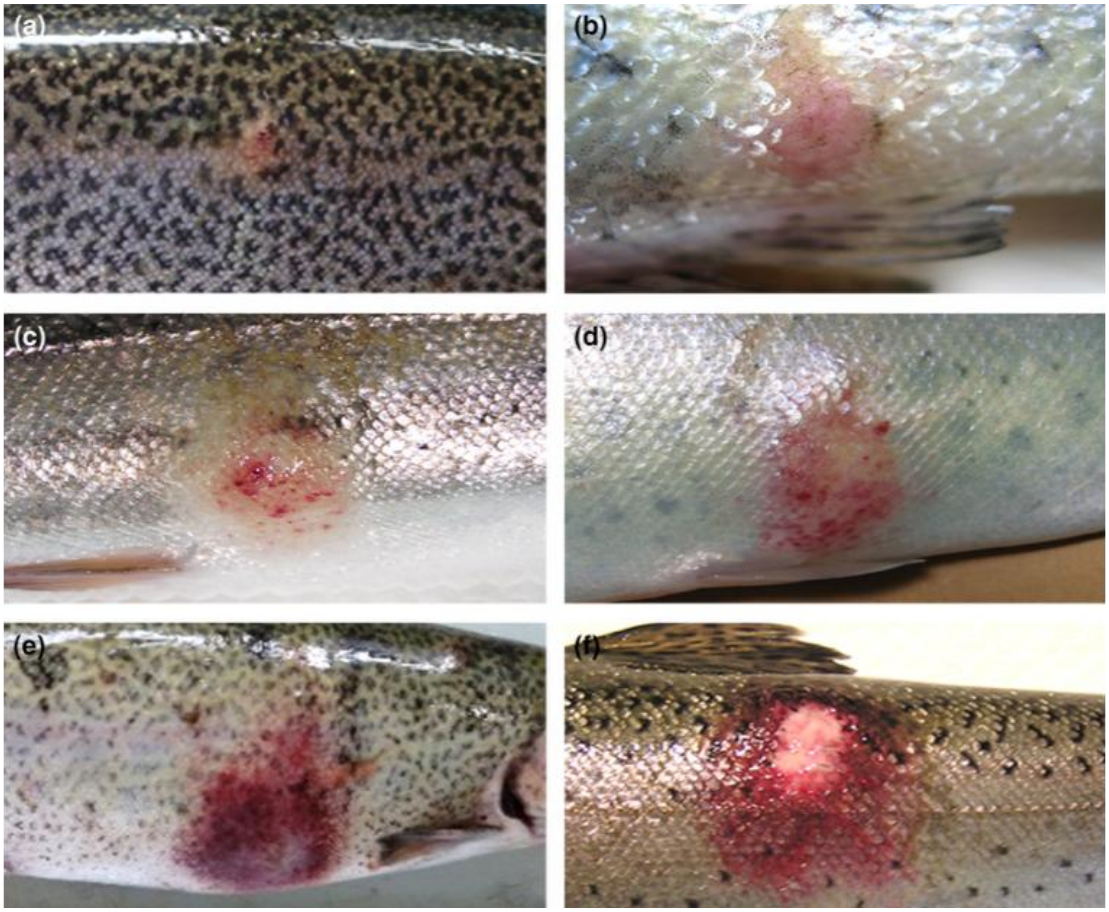



**Figure 1:** Classification of RMS skin lesions by macroscopic features into three categories. (a–b) Type I lesions, consisting of small up to 1 cm flat macules, with discoloration, mild exudate and visible scales; (c–d) type II lesions, characterized by slightly raised 1–2 cm patches, covered by clear exudate associated with multifocal petechiae and slight scale loss; (e–f) type III lesions,consisting of slightly raised large patches, more than 2 cm, covered by serous/fibrin exudate with marked redness and haemorrhages, evident scale loss and area of erosion

### 3.2 Histology

The histological observation of skin sections allowed describing the main features of each gross lesion type, as follows:

#### 3.2.1 Type I

Mild gross skin lesions. The epidermis was always intact, only rarely showing areas with slight intra-epithelial lymphocyte exocytosis. The single constant observation was a mild-to-moderate infiltration

of lymphocytes and monocytes in the SS of the dermis, involving also the area surrounding the SP. The dermal SC in some samples (5 out of 7, 71%) appeared with a mild-to-moderate lymphocyte infiltration, whereas the hypodermis revealed a moderate infiltration of lymphocytes and monocytes (Figure 2).

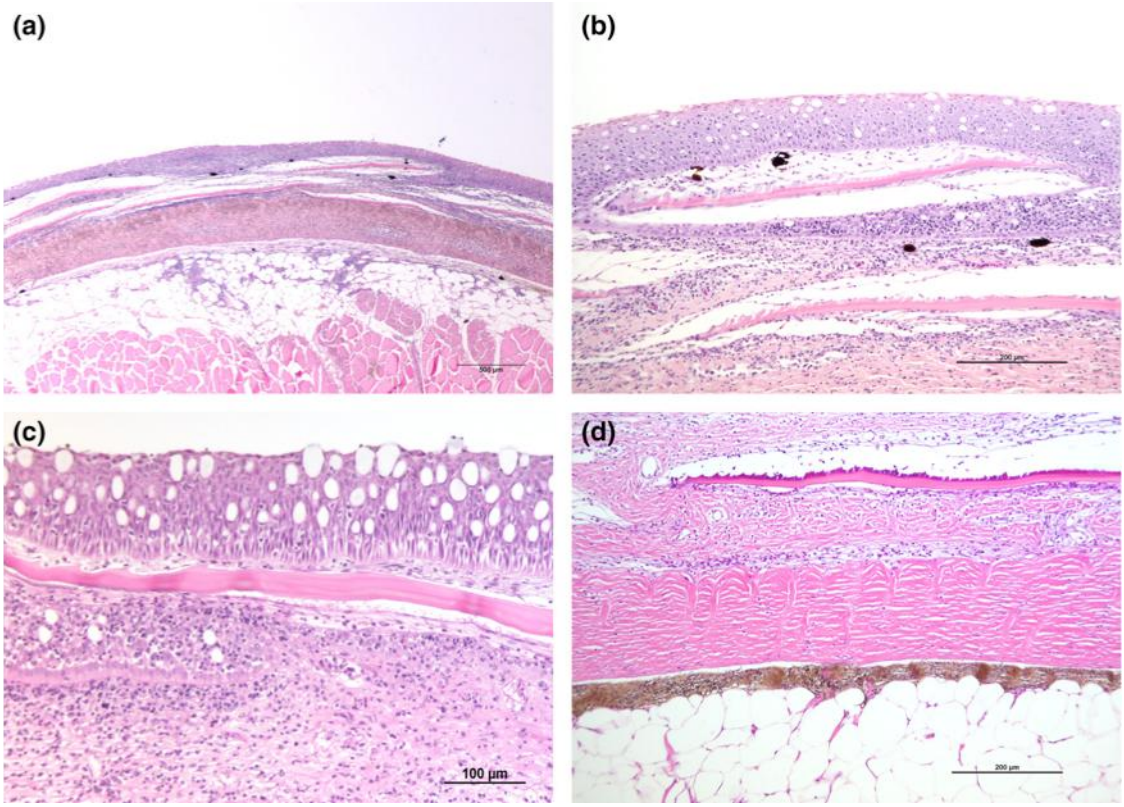



**Figure 2:** Histological features of the type I lesion. (a) Histology revealing a © inflammation involving all the skin layers, including the hypodermis. The epidermis is intact. The scale pockets and scales are present, with a © cellular infiltration (H&E). (b) Detail of Figure 2. The epidermis is still intact and the stratum spongiosum shows a moderate cellular infiltration, particularly evident under the scales (H&E). (c) Detail of the epidermis and spongiosum layer of the dermis. The

epidermis is intact and unaffected by inflammatory infiltrate, otherwise a moderate mononuclear infiltrate is observed in the spongiosum layer, mainly around scale pockets. (H&E). (d) Detail of the spongiosum layer in dermis, affected by a cellular inflammatory infiltrate. The compact layer and the hypodermis are unaffected (H&E)

### 3.2.2 Type II

Moderate gross skin lesions. There was moderate inflammation involving all the skin layers from the epidermis to the underlying muscular tissue (Figure 3°). The epidermis was often present or partially missing. In some specimens (7 out of 19, 37%), a multifocal epidermal necrosis or epidermal oedema with acanthosis was evident (Figure 3b). Multifocal mild lymphocyte intra-epithelial infiltration was observed (Figure 3b). A lichenoid inflammatory pattern was observed, characterized by monocyte and lymphocyte infiltration in the dermo-epidermal junction, sometimes associated with epidermal layer detachment (Figure 3d). The dermo-epidermal junction showed in some cases extended thickening of the basement membrane (Figure 3c). The SS was affected by moderate-to-severe infiltration of lymphocytes and monocytes as well as occasional recruitment of neutrophils and constant oedema coupled with lymphatic dilatation. SPs were frequently affected by a moderate-to-severe infiltration of lymphocytes, monocytes and macrophages distributed internally throughout the pockets or below the scales (Figure 3e). Sometimes the SPs were filled with pale eosinophilic fluid, which caused a marked oedema. Occasionally, the scales were partially or fully degenerated with multinucleated osteoclasts involved in scale resorption (Figure 3f, g). The SC appeared thickened by about a third compared with the normal thickness, with moderate-to-severe infiltration of lymphocytes (Figure 3h), slight haemorrhages and oedema. The hypodermis showed panniculitis with a moderate-to-severe infiltration of lymphocytes, plasma cells and macrophages

(Figure 3h). A mild fibrosis was also detectable. The underlying muscular tissue showed moderate-to-severe infiltration of lymphocytes and plasma cells, with frequent mitoses. Muscle inflammation was mainly localized beneath the areas of panniculitis and mainly involved the myosepta. In a few cases, both hypodermal tissue inflammation and muscular tissue inflammation showed a granulomatous pattern. Inflammation was sometimes observed in deeper areas, along the intramuscular septa and up to the peritoneum.

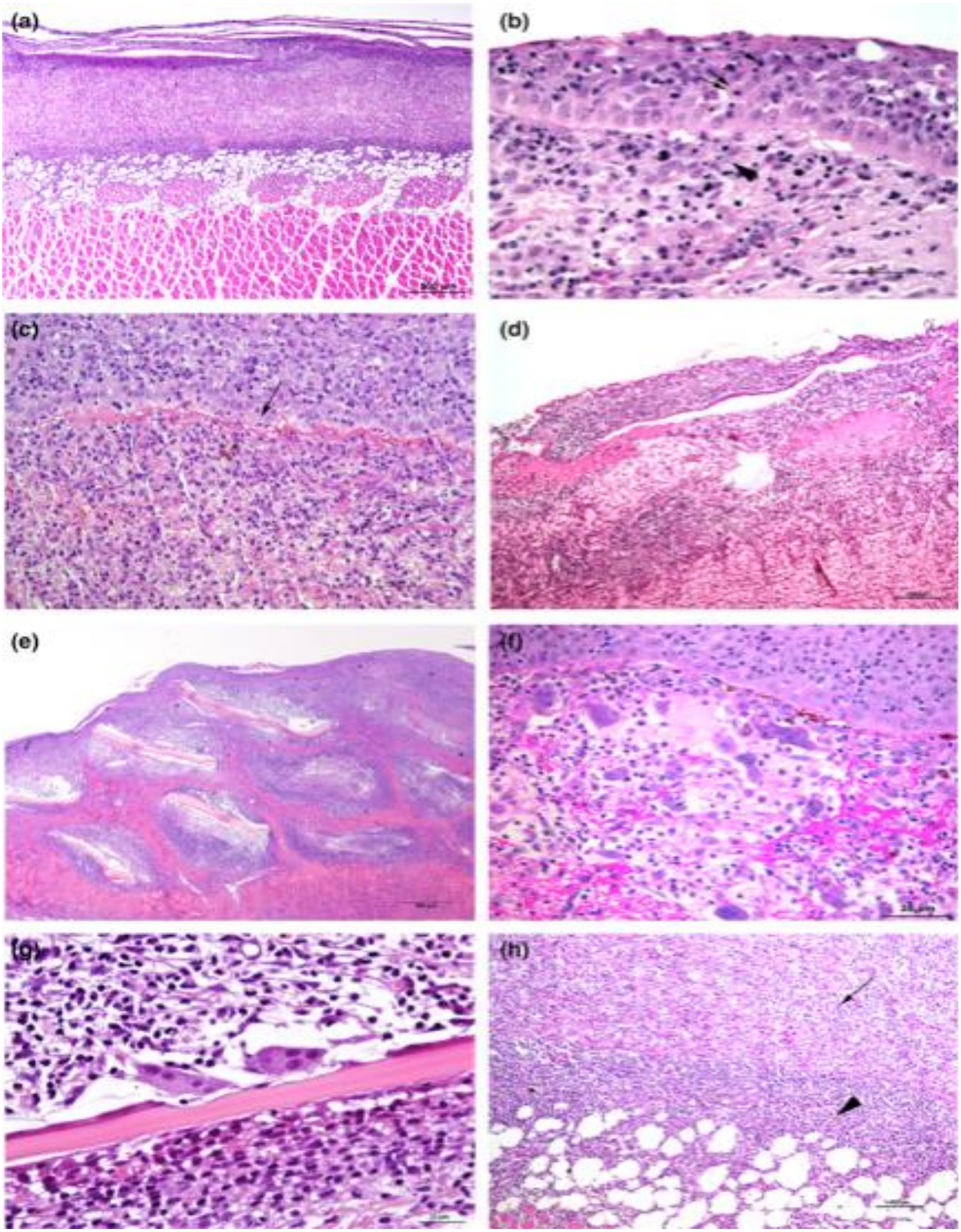



**Figure 3:** Histological features of the type II lesion. (a) Histology revealing an inflammatory response involving all the skin layers from epidermis to hypodermis, until the underlying muscular tissue. The *stratum spongiosum* appears moderately infiltrated, mainly in the areas below the scales,

which are still intact. The *stratum compactum* of the derma appears thickened with a moderate inflammatory infiltrate, recruited also towards the hypodermis and the muscular layer (H&E). (b) Detail of epidermis, early spongiosis, nuclei pyknosis (double arrow) and some infiltrating lymphocytes (single arrow) among necrotic cells. Karyorrhectic cells (arrowhead) can be observed along a mild monocyte–macrophage infiltrate in the underlying *stratum spongiosum*, in the proximity of the dermo-epidermal junction (H&E). (c) Marked epidermis thickening with a discrete inflammatory infiltrate and cell necrosis. The dermo-epidermal junction shows extended thickening of the basement membrane (arrow). Moderate-to-severe inflammatory infiltrate and necrosis in the *derma spongiosum* (H&E). (d) Severe infiltrate by mononuclear cells at the dermo-epidermal junction, with extensive necrosis characterized by abundant eosinophilic material. Evident lichenoid tissue reaction with epidermal detachment (H&E). (e) *Stratum spongiosum* shows scale pockets massively enlarged, with severe lymphocytes and monocyte infiltrate, and abundant acidophilic necrotic debris. Scales are either lacking or, when present, are under re-absorption (H&E). (f) Scale pocket with numerous multinucleated osteoclasts. The infiltrate includes macrophages and presence of acidophilic fibrillar (necrotic) or pale (oedema) material (H&E). (g) Close-up of a scale within its pocket showing an area of re-absorption determined by osteoclasts. Note abundant infiltrate by mononuclear cells both above and below the scale (H&E). (h) Transition area between the *stratum compactum* (arrow) and the hypoderma (arrowhead). Dermatitis and panniculitis with widespread and severe infiltration of lymphocytes, plasma cells and monocytes, both in the *stratum compactum* and in the hypodermis, determining a loss of architecture (H&E)

### 3.2.3 Type III

Severe gross skin lesions. A severe inflammation involving all the skin layers from the epidermis down to the underlying muscular tissue was always present (Figure 4°). The epidermis was absent

in 10 out of 20 (50%) skin sections due to erosion and ulceration of the epithelium (Figure 4°). When the epidermis was present, necrosis and severe intra-epithelial lymphocyte infiltration were observed.

At the dermo-epidermal junction, cells necrosis, capillary congestion and haemorrhages were observed. In the case of skin ulceration, the lesion involved the epidermis, the SS and partially the SC. The SS contained numerous necrotic cells and a severe inflammatory infiltrate composed of lymphocytes, macrophages and scarce neutrophils. Vascular congestion was always severe, accompanied by moderate haemorrhages and slight oedema. In some samples, the vessels displayed thrombosis (Figure 4c). Scales were absent due to complete resorption, and in some specimens, an early process of regeneration was detected. The SPs were numerically reduced or lacking, replaced by areas of chronic inflammation (recruitment of lymphocytes, plasma cells, macrophages and multinucleated giant cells) (Figure 4b). The SC displayed a severe cellular infiltrate including monocytes, macrophages, lymphocytes and plasma cells. Inflammatory cells spread between the connective fibres, modifying the tissue structure and inducing marked thickening of the skin layer as shown by Van Gieson Weigert trichrome staining (Figure 4d). A strong vascular congestion was often seen. Panniculitis often presented a granulomatous pattern, mainly concentrated around small vessels and nerve fibres (Figure 4e, f). In samples where the granulomatous pattern was more evident, multinucleated giant cells were also observed (Figure 4f). A similar pattern was present in the underlying muscular tissue. Necrosis of the myofibrils was often observed and associated with macrophages engulfing necrotic muscular tissue debris (Figure 4g). Within this histological pattern, several infiltrating cells were apoptotic. Occasionally, the phlogosis reached the peritoneal serosa, mainly in specimens where the gross skin lesions affected the flank or the ventral area. In these specimens, fibrous adhesions were detected between the serosa and the gut wall (Figure 4h).

Lesions affecting the internal organs were observed in trout with type II and type III lesions. Among the internal organs, histological lesions were observed in the heart, spleen, kidney and liver. In 19 out of 46 examined fish, heart lesions included pericarditis (17 fish, 37%) and myocarditis (2 fish, 4%), both with lymphocyte infiltration. In the spleen, hyperplasia of the white pulp associated with perivascular histiocytic cell proliferation was observed in 18 out of 46 fish (39%). The liver showed foci of lymphocyte infiltration and lympho histiocytic vasculitis in 13 out of 46 specimens examined (28%). In 14 out of 46 kidney specimens (30%), a mild vacuolar degeneration of tubular epithelium was observed. No pathological changes were observed in the gill, brain and gut samples.

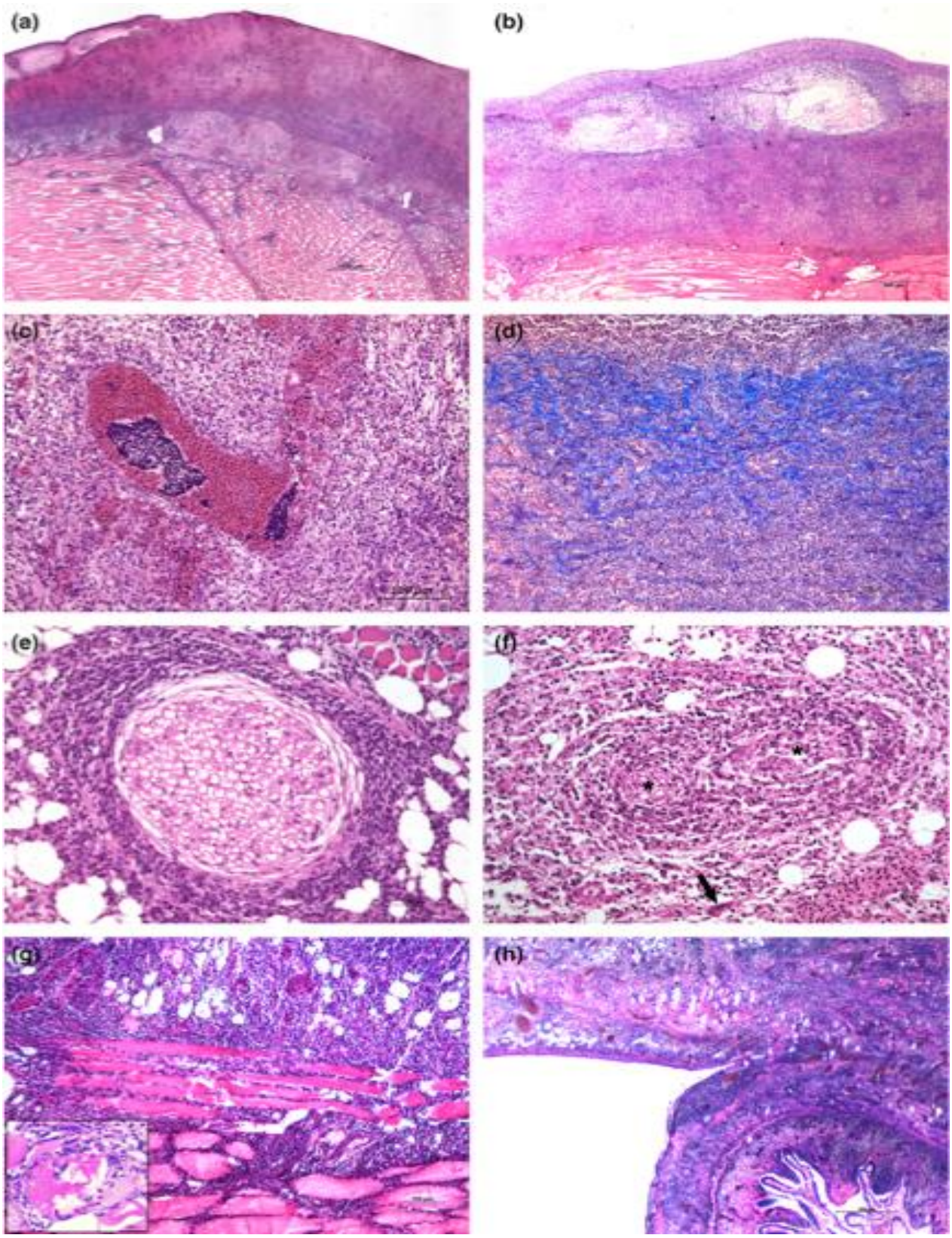



**Figure 4:** Histological features of the type III lesion. (a) Severe cellular infiltration involving all the layers from epidermis down to the muscular tissue with epidermis partially missing. The *stratum spongiosum* is thinned, and no scales can be observed. The dermal *stratum compactum* appears thickened and severely infiltrated, deeply modifying the layer's architecture. Severe infiltration

reaches the hypodermis (panniculitis) and the underlying muscular layers (myositis), spreading also between the myosepta (H&E). (b) Severe cellular infiltration pattern involving all skin layers. The epidermis is still present but subjected to lymphocyte exocytosis. Scales are absent in the two scale pockets of the *stratum spongiosum* replaced by severe oedema and influx of inflammatory cells. The *stratum compactum* is markedly thickened and heavily infiltrated (H&E). (c) High magnification of a strongly congested venous vessel in the *stratum spongiosum*, with the development of luminal thrombi and surrounded by severe infiltration (H&E). (d) Evident thickening of the skin layer due to severe and widespread cellular infiltration (monocytes, macrophages, lymphocytes and plasma cells) of the connective fibres matrix of the *stratum compactum* that modifies the tissue structure (Van Gieson Weigert trichrome). (e) Severe infiltration of lymphocytes and plasma cells surrounding nerve fibres observed as a particular feature of the panniculitis. The reaction includes a fibro-connective tissue reaction around the perineurium (H&E). (f) Granulomatous reaction surrounding residual nerve fibres (asterisks) includes numerous monocytes–macrophages, lymphocytes, histiocyte-like cells and, occasionally, multinucleated giant cells of the syncytial type (arrow) (H&E). (g) Presence of a severe lymphocyte–plasma cell infiltrate between the myofibres, inducing atrophy and loss of architecture. Insert: details of a degenerated myofibre with internal macrophages undergoing phagocytosis (H&E). (h) Fibrous adhesion reaction between the serosa and the gut wall was observed in section of the abdominal wall, intestine adherent to the serosa/muscular layer. Note inflammation in this case reaches the peritoneal serosa (H&E).

### 3.3 Data analysis by statistics

The correlation between the single histological scored parameter and the three types of gross lesions, assessed with the Spearman rank-order correlation, revealed several significant results but

with different conclusive considerations (Table 2). A significant positive correlation (score increase) from type I to type III gross lesions was apparent with the following histological parameters (supplementary graphs a–f): progressive loss of the epithelium and scales, progressive recruitment of inflammatory cells in the SC of the dermis, loss of regular architecture of the SC of the dermis and increase in granuloma-like reactions surrounding the small vessels and muscular fibres (highest R value in Table 2). The above-mentioned variables thus acquired a higher score parallel to the progression of the macroscopic lesion. For several other variables, the results showed lower values in the type I group compared with the other groups, in which type II was similar to type III. These variables did not provide any indication of the progression of the lesion from type I to type III gross features; if scored with lower values, they only offered an indication of an early and mild gross feature.

### 3.4 Immunohistochemistry

Several PCNA-positive cells were found in the epidermis (lymphocytes) (Figure 5a, b) and in the dermis (lymphocytes, fibroblasts and endothelial cells) (Figure 5c), but only PCNA-positive lymphocytes (Figure 5d) were found in the hypodermis. A significant increase in PCNA-positive lymphocytes from gross type I to gross type III cases was revealed only in the hypodermis (supplementary graph g); no other association was apparent.

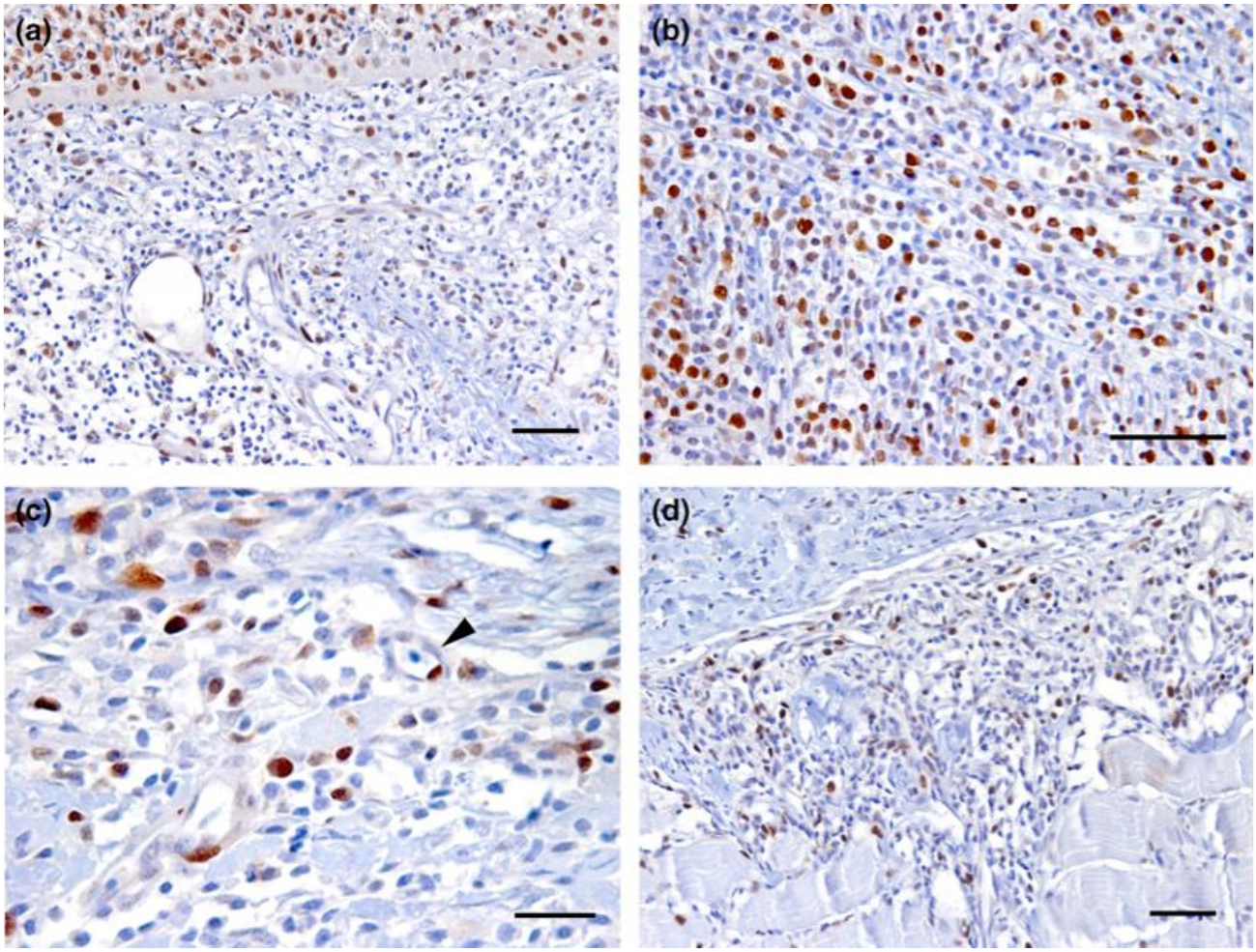



**Figure 5:** Immunohistochemical stain to PCNA. (a) Positive nuclear stain to PCNA in epidermis (top of the figure) and dermis, in this latter localized mainly in lymphocytes. (b) Higher magnification of dermal-positive nuclear stain to PCNA. (c). Positive nuclear stain to PCNA in vessels (arrowhead). (d) Subcutis showing positive PCNA lymphocytes. Bar: a, b, d = 50 micron; c = 25 micron. All images from type 3 lesions.

## 4. Discussion

The clinical evaluation and the differential characterization of theskin lesions examined in the present study were based on the RMS case definition in rainbow trout proposed by Oidtmann et al. (2013). The cases analysed here were compatible with RMS considering the criteria suitable for the

definition of RMS outbreaks such as affected fish size, water temperature, clinical signs, gross lesion appearance and distribution and histopathological features. Moreover, part of the skin lesion samples analysed confirmed the presence of intracytoplasmic microorganisms resembling Rickettsiales within macrophages, fibroblasts and erythrocytes by TEM, as described previously by Galeotti, Manzano, et al. 2017. From a macroscopic point of view, the skin lesions of the cases described here do not differ from those observed in the United States as USSD (Lloyd et al., 2008; Olsen et al., 1985) and in other European countries (Ferguson et al., 2006;, Oidtmann et al., 2013; Noguera et al., 2008; Rodger, 2008; Schmidt et al., 2017; Schimidt-Posthaus et al., 2009) or Turkey (Kubilay et al., 2014). From the results of histological evaluations, type I lesions are characterized by an always intact epidermal layer, a slight lymphocyte and monocyte infiltration in the dermal SS, also involving the area surrounding the SP, and a scarce presence or complete absence of neutrophils. Type II lesions are characterized by inflammation involving all the skin layers, from the epidermis to the underlying muscular tissue, a moderate-to-severe inflammatory reaction on the scales and the SP with mononuclear cell infiltration, both within the pockets and right below the scales. The scales are partially or fully degenerated with multinucleated osteoclasts, which are involved in scale resorption. The hypodermis shows a moderate infiltration of mononuclear cells. In type III lesions, scales are absent due to complete resorption, the SC is markedly thickened with loss of normal structure due to evident lymphocyte and monocyte infiltration and myofibril necrosis is often observed. These findings are consistent with those reported previously by other authors (Ferguson et al., 2006; Fleury et al., 1985; Kfoury et al., 1996; Kubilay

et al., 2014; La Patra et al., 1994; Lloyd et al., 2008; McCarthy et al., 2013; Metselaar et al., 2010; Noguera et al., 2008; Olson et al., 1985; Rodger, 2008; Sasani et al., 2016; Sandoval et al., 2016; Schmidt Posthaus et al., 2009; Verner-Jeffreys et al., 2008). The term “panniculitis” denotes the localization of inflammation to the fat of the subcutis. From a comparative point of view, the

existence of a similar stratigraphy (skin/subcutis/muscle) in mammals and fish allows the use of the term “panniculitis” in fish. It results from primary damage of subcutis or by extension from surrounding layers, mainly secondary to dermatitides. Panniculitis from any cause is enhanced by lipid release from damaged lipocytes because lipids are vigorous stimulators of inflammation (Gross et al., 2005). Adding to the current state of knowledge, the results of the present work contribute particularly with the observations of type II and type III lesions; these data highlight that panniculitis and myositis may display a granulomatous pattern surrounding vessels as well as the nerves and muscle fibres. This pattern, when observed in the hypodermis, can be defined as panniculitis referring to the development of severe granulomatous reactions in the proximity of blood vessels and nerve fibres. This condition is consistently associated with severe myositis of the underlying tissue. Furthermore, in some samples, the chronic inflammatory reaction also reaches the peritoneal serosa, with adhesions developing between the serosa and the gut wall. Furthermore, the SS of type III lesion in some samples shows vessels with occlusive thrombosis and early processes of scale regeneration, thus demonstrating that this regeneration occurs not only in fully healing injuries but also in ongoing injuries. At the internal organ level, the most relevant microscopic lesions were observed in the heart and spleen. The pericarditis observed in 17 out of 46 trout affected by RMS represented the most prominent lesion, characterized by an intense lymphocytic infiltration of the pericardium. Similar findings were described by Ferguson et al., (2006), who reported heart lesions in 40% of SD-affected fish (20% of which showed diffuse myocarditis) and by Verner-Jeffreys et al., (2008), who reported epicarditis and cardiomyositis in 20% of the examined fish with RMS. In the spleen, hyperplasia of the white pulp associated with perivascular histiocytic cell proliferation was observed in 18 out of 46 individuals (39%), as also described by Metselaar et al., (2020). In 13 out of 46 trout examined, the liver showed foci of lymphocyte infiltration and vasculitis. The observation of liver focal necrosis was also recorded in RMS-

affected fish by Noguera et al., (2008) and Verner-Jeffreys et al., (2006). Mild tubular degeneration in the kidney was observed in 14 out of 46 specimens, similarly to findings by Noguera et al., (2008) and Verner-Jeffreys et al., (2006), whereas a moderate-to-severe diffuse histiocytic proliferation of the interstitial haematopoietic tissue was also reported by Metselaar et al. (2010) and Metselaar et al. (2020).In the gill, brain and gut, no pathological changes were observed. All the lesions in the internal organs were recorded in trout showing macroscopic lesions classified as type II or type III, suggesting a systemic compromise. The observations made in this study and the statistical data analysis allowed us to survey the evolution of the histological lesion and to define three macroscopic stages identifying the most relevant phases of the disease progression. The correlation between single histologically scored parameters with the three gross lesion types revealed meaningful correlations for some variables. A significant positive correlation (score increase) from type I to type III gross lesions was apparent in the case of six parameters: progressive loss of the epithelium, progressive loss of the SP, progressive recruitment of inflammatory cells in the SC of the dermis, loss of regular architecture of the SC of the dermis, increase in granuloma-like reactions surrounding the small vessels and nerve fibres of the hypodermis and in the muscular fibres. The above-mentioned variables thus acquire a higher score, parallel to the progression of the macroscopic lesion. The observation of these lesions together with the recovery of vessels displaying occlusive thrombosis in SS indicates a clear aggravation of the process in the skin. It is also appropriate to take into consideration the observation of pericarditis and of splenic reactivity. These data could be used for an objective assessment of the aggravation and systemic spread of the process, beyond the morphological aspect of the external lesions.

Regarding some possible insights on the disease pathogenesis, our observations are in line with those reported by Ferguson et al., (2006). Skin lesions display aspects similar to the lichenoid dermatitis observed and described in humans and other mammals as a result of the T-cell-mediated or autoimmune response (Deschaine & Lehman, 2019; Gross et al., 2005). The strong presence of

lymphocytes and macrophages, also reported by McCarthy et al., (2013) and von Gersdorff Jørgensen et al., (2019), suggests an immunopathological mechanism promoting the development of these lesions. In our study, the presence of microthrombosis and necrosis of the epidermis observed in advanced severe lesions is similar to that recorded in skin inflammatory reactions caused by the accumulation of immune complexes (type III), which can coexist with forms of delayed-type hypersensitivity (type IV), as happens in canine scabies, where type III and type IV mechanisms coexist (Mauldin & Peters-Kennedy, 2016). Furthermore, the fact that skin lesions caused by RMS easily tend to recover spontaneously reinforces the hypothesis of an immune-mediated disease, as observed in serum sickness, an experimental animal model of human immunological disease (Lawley et al., 1984; Rixe et al., 2020). Recent studies on RMS pathogenesis have suggested the involvement of a delayed type Th-1 cell-mediated immune response with a concurrent high production of immunoglobulins IgD, IgM and IgT, possibly independent from the Th-2 pathway (von Gersdorff Jørgensen et al., 2019). Concerning the immunohistochemical detection of PCNA positivity, it is interesting to note how positive proliferating cells were found in the epidermis (only lymphocytes), the dermis (lymphocytes, fibroblasts and endothelial cells) and the hypodermis (only lymphocytes). A significant increase in PCNA-positive lymphocytes from type I to type III lesions was only observed in the hypodermis. The proliferation model in endothelial cells (which indicates angiogenesis) and in fibroblasts (which indicates tissue repair) is consistent with an inflammatory reaction localized in the dermis, whereas the presence of proliferating lymphocytes, associated with granulomatous inflammation, indicates lymphocyte activation in the hypodermis. In conclusion, in the light of our experience with the histological observation of skin lesions from 46 RMS-affected trout, classified in three gross developmental stages, and based on the statistical analysis of the histological scores, we suggest an improvement of the initial first accurate descriptions of the “Strawberry disease” skin lesion “...focal, non-suppurative dermatitis with ulceration and extensive infiltration of

mononuclear inflammatory cells" (Olson et al., 1985), proposing a new morphological diagnosis as "...deep chronic dermatitis associated to panniculitis and myositis, character ised by lympho-histiocytic and granulomatous reaction."

# 5. Development and application of a sensitive droplet digital PCR for the detection of red mark syndrome infection in fish spleen

## 1. Introduction

The RLO, belonging to the Midichloriaceae family and thus referred as MLO (Montagna et al, 2013; Cafiso et al. 2016), is currently considered the strongest etiological candidate for RMS (Metselaar et al, 2020). It has been consistently detected by IHC (immunohistochemistry), PCR (polymerase chain reaction), nested PCR and qPCR (quantitative real-time PCR), in at least three different geographical areas (Metselaar et al, 2022). PCR and nested PCR techniques have some disadvantages including relatively low sensitivity and the need to use agarose gel electrophoresis to visualize the amplification products. This can generate DNA products contamination. Real time PCR is a quantitative detection method with an higher sensitivity and specificity. It does not require the use of gel electrophoresis. To produce relative quantification a reference gene is required, while for absolute quantification, a standard curve is needed. Moreover, qPCR could display low accuracy in samples with low bacterial loads, such as water samples, eggs, larvae and fry (Lin et al. 2020).

When compared with the above PCR methods, droplet digital polymerase chain reaction (ddPCR) has been shown to generate accurate results in low copy number quantification and is less susceptible to PCR inhibitors (Gutiérrez-Aguirre et al., 2015; Li et al., 2018). This technique has been used in the detection of bacteria, such as *Flavobacterium psychrophilum* and *Yersinia ruckeri* in Norwegian aquaculture systems water (Lewin et al., 2020), virus, such as spleen and kidney necrosis virus (Lin et al., 2020) and Singapore Grouper Iridovirus (Yuan et al., 2016) and parasites, such as *Gyrodactylus salaris* in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Rusch et al., 2018).

To the authors' knowledge, this is the first report of its use for the detection of MLO in rainbow trout.

## 2. Materials and methods

### *2.1 Primers and probe*

The primers and probe selected derived from Lloyd et al., 2011 and recognize the sequence registered in GenBank as accession number EU555284 (Lloyd et al., 2008) (Table 1).

The probe was labeled with Fluorescein (FAM) as fluorescent reporter and Black Hole as fluorescence quencher (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The primers were synthetized by Invitrogen (Thermo Fisher Scientific). The same primers and probe were used in both ddPCR and qPCR reactions.

### *2.2 Optimization of the ddPCR method*

The ddPCR method was performed in a 20 μL reaction volume, in accordance with the manufacturer's instructions (Bio-Rad, USA). Briefly, the reactions included 10 μL of 2× ddPCR™ Supermix No-UTP for Probes (Bio-Rad, USA), 1 μL of each forward and reverse primer (10 μM), 1 μL of probe, 7 μL of DNase/RNase-free $H_2O$ and 1 μL of DNA template (corresponding to 50 ng of total DNA). The reactions were optimized for probe concentration (125, 250, 500, 750, 1000 and 1500nM). The reaction mixture (20 μL) for each sample was loaded into a well of a disposable DG8™ cartridge (Bio-Rad, USA) and 70 μL of Droplet Generation Oil (Bio-Rad, USA) were placed into each of the adjacent oil wells in the cartridge (Bio-Rad, USA). Droplets were produced in each well using a QX200™ droplet generator (Bio-Rad, USA). The droplets were then transferred to a 96-well PCR plate (Bio-Rad, USA). To differentiate the amplitude between the

negative and positive droplets and to reduce the background of the negative droplets, we performed a temperature gradient in the annealing step. The PCR amplifications were performed with an initial step of 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 64.5 °C for 60 s and 1 cycle of 98 °C for 10 min, with a final hold at 4 °C. To achieve the best results for the method, a range of annealing temperatures (58, 58.5, 59.4, 60.8, 62.5, 63.9, 64.5 and 65 °C) was tested. After PCR amplification of the DNA target in the droplets, the microdroplets from each well were read individually using a QX200 Droplet Reader (Bio-Rad, CA, USA). A threshold was set between the positive and negative microdroplet clusters and the copy number of each well was evaluated automatically by QuantaSoft™ version 1.7 (Bio-Rad, CA, USA).

A post-run analysis was adopted to evaluate the quality of the data produced by the ddPCR reaction. The preliminary quality control was the count of total number of droplets generated with ≥ 7,000 droplets/well. To ensure an accurate classification of compartments and thus a reliable quantification of positives and negatives droplets, three different criteria were adopted.The first requirement was to set a quantification threshold that would allow the production of a single amplicon per signal. The second aspect was the definition of the best peak resolution; in order to produce the widest signal separation. The third criteria was based on the removal of stragglers or 'rain' droplets that have an intermediate fluorescence and do not belong to either the positive or negative population.

**Table 1:** Primers and probe used in the study.

| Gene | Sequence name | Sequence (5'-3') |
|---|---|---|
| MLO | Forward | GGCTCAACCCAAGAACTGCTT |
| | Reverse | GTGCAACAGCGTCAGTGACT |
| | Probe | CCCAGATAACCGCCTTCGCCTCCG |

*2.3 Real-time PCR method*

The qPCR amplification reactions were performed in a final volume of 20 μL, which contained 1× iQ Supermix (Bio-Rad, Hercules, CA, USA), 400 nM each primer, 250 nM probe, DNA (1 μL) and $H_2O$ (6.9 μL). The qPCR reactions were performed using the CFX96 real-time PCR System (Bio-Rad) as follows: denaturation at 95°C for 3 min, 40 cycles of 95°C for 30 s and 64.5 °C for 60 s. Fluorescent signals were collected during the 64.5°C step of each cycle.

*2.4 Specificity of the ddPCR method*

To evaluate the specificity of the MLO-specific ddPCR method, DNA from the fish affected with RMS and DNA from bacteria strains of *Lactococcus garvieae*, *Yersinia ruckeri*, *Photobacterium damselae piscicida* and *Vibrio anguillarum* were tested.

Sterilized water was used as negative control. These bacteria strains were stored at the Veterinary Pathology laboratory of Udine University. The authors cultivated the bacteria using standard media and conditions, and performed the DNA extraction by QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following their standard protocol for bacteria.

*2.5 Analytical sensitivity of ddPCR and qPCR methods*

To evaluate the sensitivity of the two methods, a serial diluition (1:1; 1:2; 1:5; 1:10; 1:50; 1:100) was prepared starting from a MLO positive samples derived from the RMS experimental infection model established at the DTU-Aqua, National institute of Aquatic Resources and European reference laboratory for fish and shellfish diseases, Denmark. The same sample serial dilution was tested by qPCR and ddPCR to determine the detection limit of the two methods. Each dilution was tested in triplicate, in three independent runs, to evaluate inter-assay reproducibility.

The standard curve for the qPCR quantification was generated using a 10-fold serial dilution of the DNA of another MLO positive sample derived from DTU aqua institute. Fluorescent signals from

the FAM-labeled MLO probe in the qPCR assay were analysed by the CFX96 real-time PCR System (Bio-Rad). The qPCRs were performed in triplicate. The amplified DNA of the dilutions was purified, the concentration was determined, the number of copies was calculated, and the standard curve was constructed by plotting log of the number of copies (Log starting quantity) against the values of Ct.

*2.6 Comparison of efficacy of ddPCR and qPCR methods*

To compare the efficacy of the ddPCR method for MLO detection with that of qPCR, we collected spleen from 40 healthy and infected rainbow trout spleens obtained from the RMS experimental infection unit at the DTU-Aqua. Total DNA was extracted from tissue samples using the QIAamp DNA Mini Kit (Qiagen), in accordance with the manufacturer's instructions. The DNA was tested by ddPCR using the optimized protocol described above. The results were compared with those obtained from the qPCR method, which was performed in parallel.

*2.7 Statistical analysis*

All statistical analyses and data plotting were performed using GraphPad Prism software (Version 5.0; La Jolla, CA, USA). Kappa statistics were used to compare the detection results from the ddPCR and qPCR methods and to determine their level of agreement.

**3. Results**

*3.1 Development of an MLO-specific ddPCR method*

Initially, we assessed different probe concentrations (125, 250, 500, 750, 1000 and 1500 nM) using the ddPCR method. The results showed that the optimal concentration was 250 nM, which generated the largest number of positive droplets (Figure 1 and 2). Next, we identified the optimal annealing temperature for the ddPCR method by testing temperatures of 58, 58.5, 59.4, 60.8, 62.5, 63.9, 64.5 and 65°C. As shown in Figure 3, as the annealing temperature rose, the distinction between the intensity signals of the positive (blue) and negative (grey) droplets became bigger. The annealing temperature of 64.5°C gave the greatest distinction between the positive and negative signals, the largest number of positive droplets and the lower dispersion among the positive droplets. Therefore, 64.5°C was chosen as the optimal annealing temperature for the ddPCR method.

The ddPCR software reported the copy number concentration of each sample automatically after applying the positivity threshold that allows the production of a single amplification signal. The minimum number of droplet accepted was 7’000 droplets/well.

The Poisson error and total error were calculated by QuantaSoft software and indicate the Poisson 95% confidence intervals for each copy number determination. Results obtained were accepted when relative standard deviation values were below the 5%.

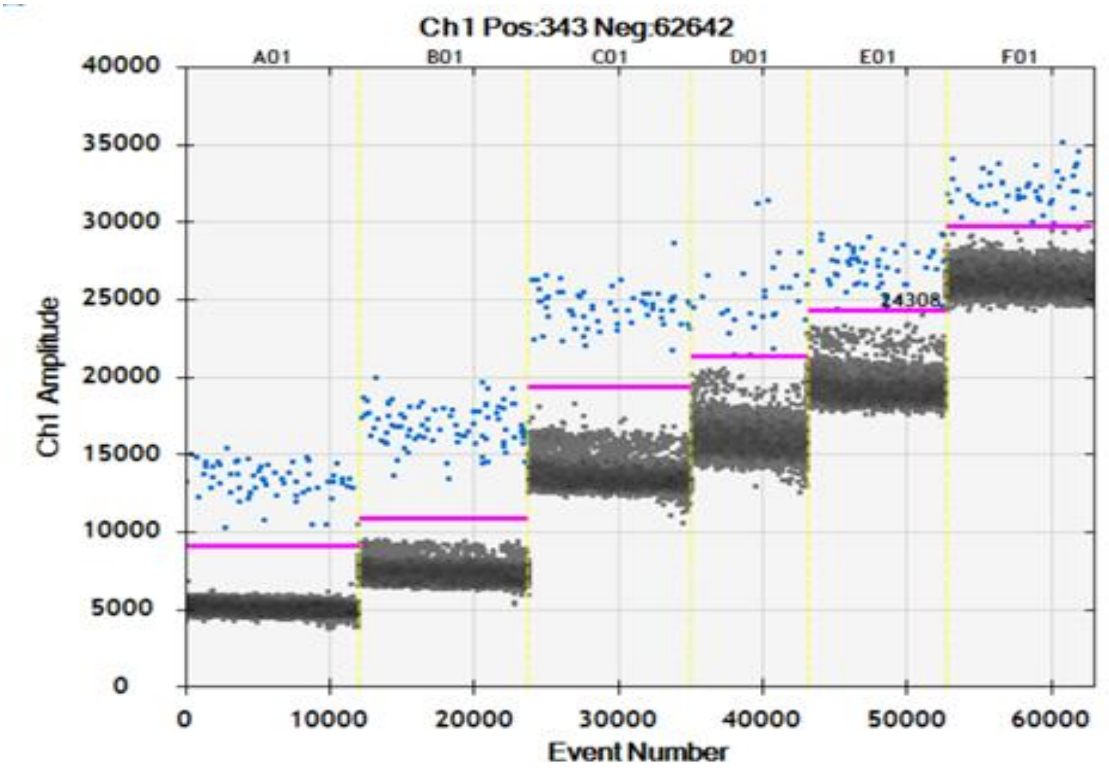



**Figure 1:** Optimization of the probe concentration for the ddPCR method. Lanes A01, B01, C01, D01, E01 and F01, which are divided by vertical yellow lines, represent the gradient of the following probe concentrations: 125, 250, 500, 750, 1000 and 1500 nM. Lane B01 (probe concentration 250 nM) showed the lower positive events dispersion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

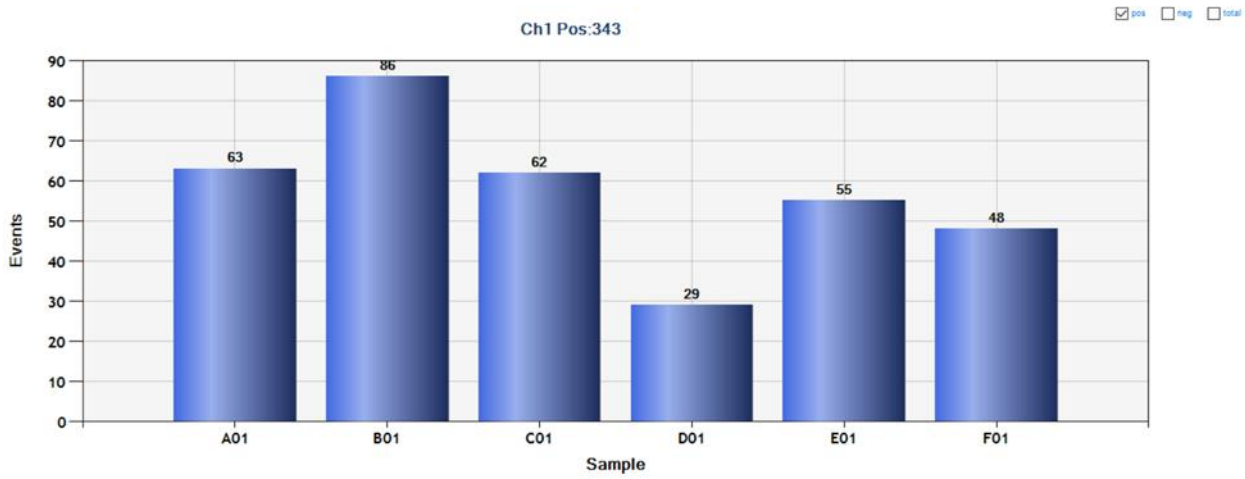


**Figure 2:** Optimization of the probe concentration for the ddPCR method. Columns A01 to F01 represent the gradient of the following probe concentrations: 125, 250, 500, 750, 1000 and 1500 nM. Lane B01 (probe concentration 250 nM) showed the higher positive events count.

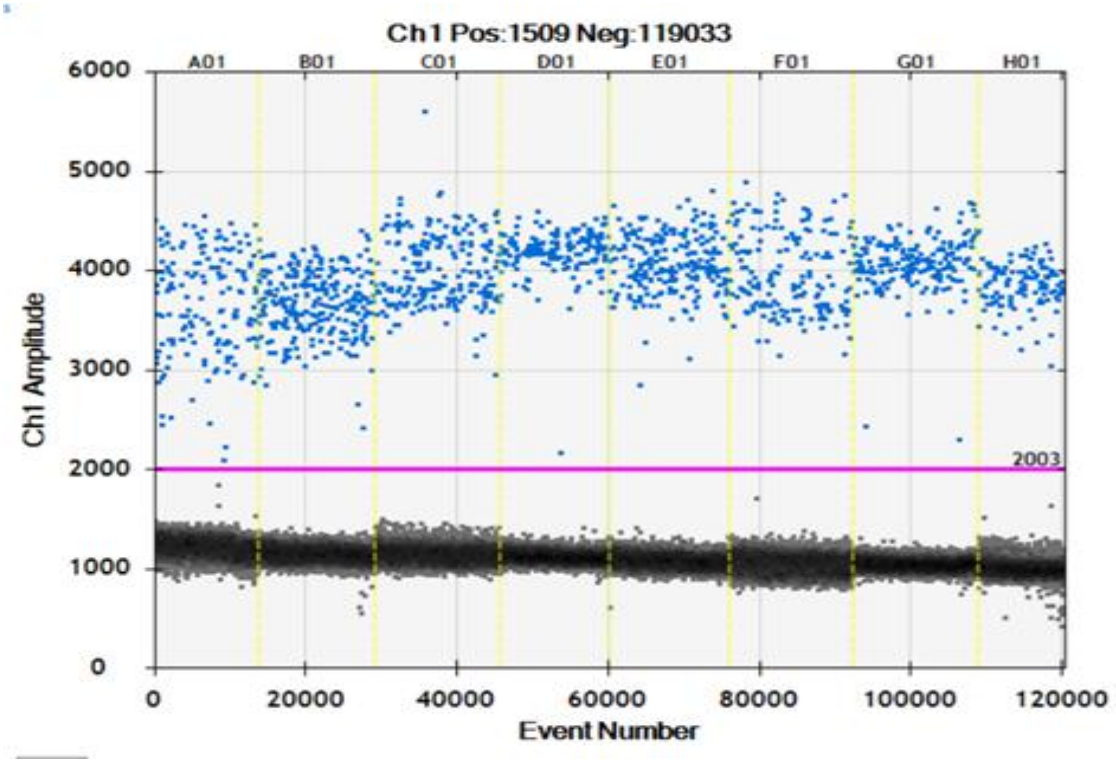



**Figure 3:** Optimization of the annealing temperature for the ddPCR method. Lanes A01 to H01, which are divided by vertical yellow lines, represent the gradient of the following annealing temperatures: 58, 58.5, 59.4, 60.8, 62.5, 63.9, 64.5 and 65°C. Lane G01 (annealing temperature 64.5°C) showed the lower positive events dispersion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

*3.2 Specificity of the ddPCR method*

To evaluate the specificity of the ddPCR method, DNA from the fish affected with RMS and DNA from four bacteria strains: *Lactococcus garvieae*, *Yersinia ruckeri*, *Photobacterium damselae*

*piscicida* and *Vibrio anguillarum* were tested. As shown in Figure 4, only MLO samples tested positive, whilst samples containing all the other pathogens tested negative. The results showed that the ddPCR method was specific for the detection of MLO.

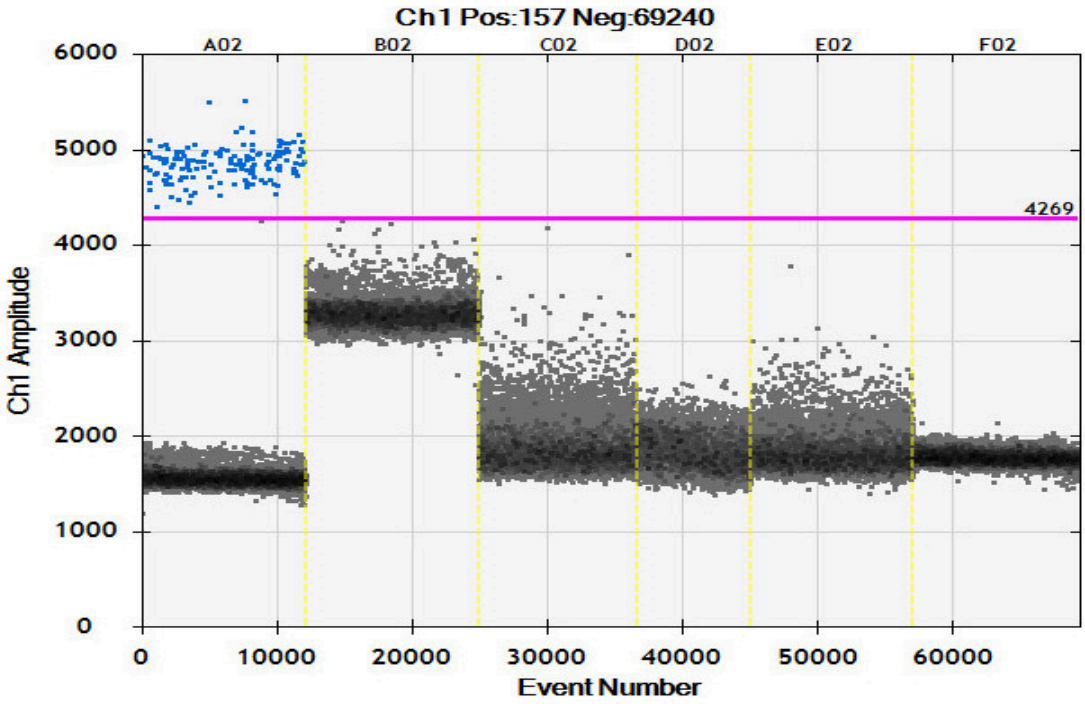



**Figure 4:** The specificity of the ddPCR method. Lanes A02, B02, C02, D02, E02 and F02, which are divided by vertical yellow lines, represent the fluorescence for MLO, *Photobacterium damselae piscicida*, *Yersinia ruckeri*, *Lactococcus garvieae, Vibrio anguillarum* and water (negative control) samples, respectively. Only MLO samples tested positive, whilst samples containing the other pathogens or water tested negative. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

*3.3 Analysis of standard curves using the qPCR method*

Fluorescent signals from the FAM-labeled MLO probe in the qPCR assay were analysed by the CFX96 real-time PCR System (Bio-Rad) and a standard curve was drawn (Figure 5). The slope of the standard curve and the PCR efficiency (99.2%) value were in the appropriate range. The equation for the regression line was $Y = -3.334\times + 41.459$. The $R^2$ value of the standard curve was 0.9943 and indicated good linearity.

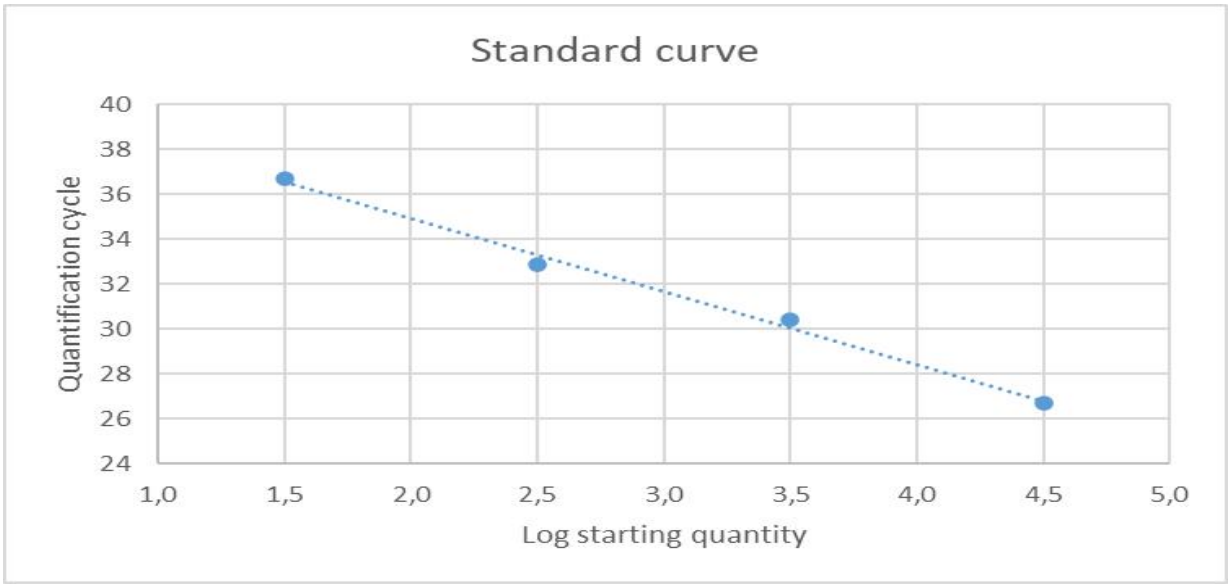



**Figure 5:** Standard curve from the qPCR method. Ten-fold serial dilutions of the DNA amplified and purified of a MLO positive sample were assessed in triplicate by the qPCR method. The quantification correlation was obtained by plotting the quantification cycle (mean of the triplicate) against the log starting concentration. The $R^2$ value was 0.9943.

*3.4 Detection limits of ddPCR and qPCR methods*

A serial dilution of the DNA of a positive sample supplied by DTU Aqua, was used to measure the detection limits of the qPCR and ddPCR methods. In the sensitivity tests, the lowest detectable concentration was 2.2 copy number for ddPCR, corresponding to 1:50 dilution of the sample and 6 copy number input, instead, the lowest detectable concentration for qPCR was 36 copy number, corresponding to 1:10 dilution of the sample and 30 copy number input (Table 2, Figure 6). The

results indicated that the established MLO-specific ddPCR method was able to detect very low concentrations of template and was considerably more sensitive (an order of magnitude higher) than the qPCR method used in our study.

**Table 2:** Sensitivity of the ddPCR and qPCR methods, obtained by the serial dilution of a positive sample DNA.

| Sample dilution | Input DNA copy number | ddPCR | | | qPCR | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 1 | 2 | 3 |
| 1:1 | 300 | 302 | 304 | 272 | 272 | 252 | 261 |
| 1:2 | 150 | 144 | 156 | 138 | 144 | 145 | 138 |
| 1:5 | 60 | 50 | 55 | 38 | 80 | 83 | 82 |
| 1:10 | 30 | 20 | 28 | 24 | 36 | 36 | 36 |
| 1:50 | 6 | 2.5 | 2 | 2 | ND | 8 | ND |
| 1:100 | 3 | ND | ND | ND | ND | ND | ND |

ND = non detected.

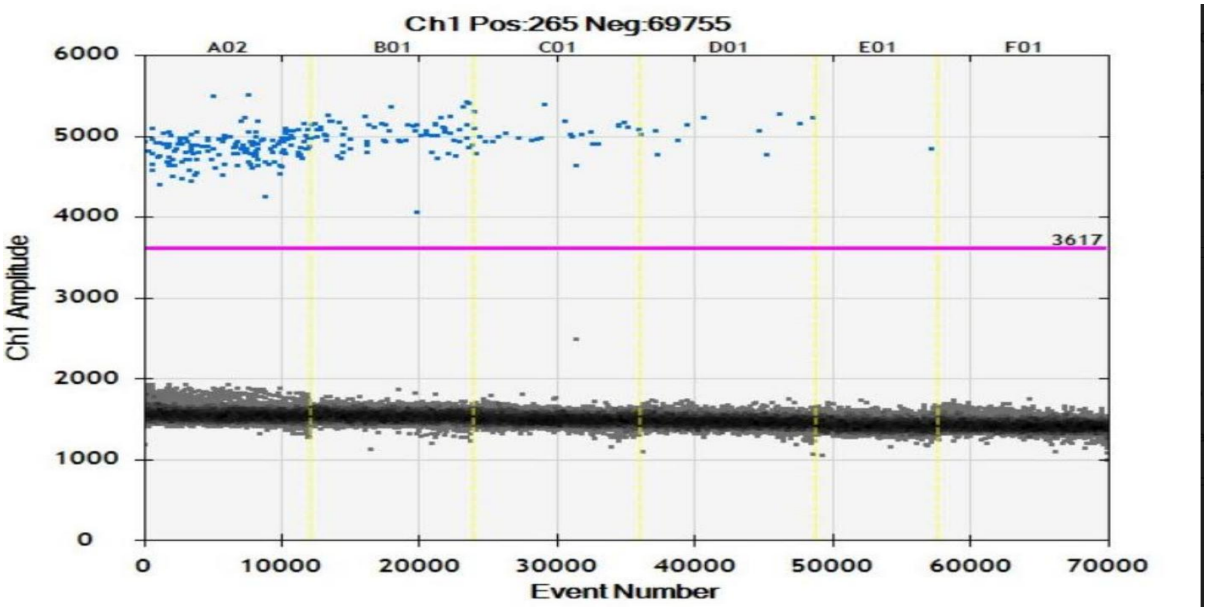



**Figure 6:** Determination of the limit of detection for the ddPCR method, one of three test. Lanes A02 to F01, which are divided by vertical yellow lines, represent the following

observed copy number: 302, 144, 50, 20, 2.5 and 0, respectively, corresponding to the following dilutions of the positive sample: 1:1, 1:2, 1:5, 1:10, 1:50 and 1:100 respectively. The lowest detectable concentration was 2.5 copy number. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

*3.5 Detection of MLO in rainbow trout spleen samples using ddPCR and qPCR methods*

The 40 samples of rainbow trout (healthy and infected) spleen were obtained from DTU aqua institute. The MLO presence in these samples was detected using both the qPCR and ddPCR methods. The detection rates were calculated, and the results are shown in Table 3. The ddPCR method gave more positive samples (65%) when compared to the qPCR method (52.5 %), which indicated that ddPCR was a more effective method for the detection of the bacteria in fish with low bacterial loads. The percentage of agreement between the two analyses was 87.5%, with a Cohen's k-coefficient of 0.75 (substantial agreement). Table 3 also shows that the bacterial load range detected by ddPCR was 2 to 606 copies whilst the bacterial load range detected by qPCR was 2 to 378 copies.

Positive droplets ranged from 0 to 285, with a mean of 13.53 and a standard deviation of 47.51. Copy number of detected DNA using ddPCR ranged from 0 to 606, with a mean value of 26.03 and standard deviation of 97.66. The ratio between detected DNA and number of positive droplets is 1.92.

**Table 3:** RLO DNA copy number in rainbow trout spleen determined by using ddPCR and qPCR.

| Sample n. | ddPCR | | | qPCR | |
|---|---|---|---|---|---|
| | Number of droplets | Positive droplets | Detected DNA | Mean Cq value | Detected DNA |
| 1 | 14034 | 6 | 18 | 37.8 | 13 |

| | | | | | |
|---|---|---|---|---|---|
| 2 | 10886 | 1 | 2 | ND | 0 |
| 3 | 13904 | 1 | 2 | ND | 0 |
| 4 | 10883 | 4 | 14 | 38.8 | 6 |
| 5 | 12973 | 7 | 11 | 39.6 | 4 |
| 6 | 10898 | 1 | 2 | ND | 0 |
| 7 | 11385 | 0 | 0 | ND | 0 |
| 8 | 13326 | 2 | 4 | 39.2 | 5 |
| 9 | 14788 | 0 | 0 | ND | 0 |
| 10 | 15279 | 0 | 0 | ND | 0 |
| 11 | 16679 | 0 | 0 | ND | 0 |
| 12 | 15645 | 0 | 0 | ND | 0 |
| 13 | 12839 | 0 | 0 | ND | 0 |
| 14 | 13603 | 0 | 0 | ND | 0 |
| 15 | 14470 | 3 | 4 | 39.7 | 3 |
| 16 | 15038 | 3 | 17 | 38.4 | 8 |
| 17 | 12333 | 2 | 6 | ND | 0 |
| 18 | 15330 | 2 | 44 | 37.2 | 18 |
| 19 | 14584 | 3 | 19 | 37.3 | 18 |
| 20 | 14949 | 2 | 4 | 40.4 | 2 |
| 21 | 16686 | 6 | 18 | 39.3 | 4 |
| 22 | 10943 | 0 | 0 | ND | 0 |
| 23 | 10295 | 0 | 0 | ND | 0 |
| 24 | 11576 | 2 | 4 | 39.6 | 4 |
| 25 | 10438 | 1 | 2 | 40.2 | 2 |
| 26 | 11738 | 5 | 10 | 39.4 | 4 |
| 27 | 9847 | 7 | 14 | 38.1 | 10 |
| 28 | 8706 | 3 | 8 | 39.7 | 3 |
| 29 | 14226 | 5 | 10 | 39.0 | 5 |
| 30 | 14390 | 86 | 192 | 36.1 | 40 |
| 31 | 14542 | 4 | 10 | 39.4 | 4 |
| 32 | 10777 | 1 | 2 | 39.4 | 4 |
| 33 | 11097 | 6 | 16 | 37.7 | 14 |
| 34 | 9054 | 0 | 0 | ND | 0 |
| 35 | 11035 | 285 | 606 | 32.9 | 378 |
| 36 | 7186 | 0 | 0 | ND | 0 |
| 37 | 9802 | 0 | 0 | ND | 0 |
| 38 | 7695 | 0 | 0 | ND | 0 |
| 39 | 9241 | 0 | 0 | ND | 0 |
| 40 | 10516 | 93 | 2 | ND | 0 |

ND = non detected.

## 4. Discussion

The ddPCR method has been used as a specific and sensitive molecular detection system in fish pathology for some bacteria, virus and parasites (Yuan et al, 2016; Rusch et al., 2018; Lewin et al., 2020; Lin et al., 2020). When compared with other detection techniques, ddPCR is advantageous as it is sensitive, accurate and does not require an external standard curve (Gutiérrez-Aguirre et al., 2015). In this study, we established a ddPCR method to detect and quantify MLO DNA loads associated with RMS in fish spleen samples. This showed high specificity and sensitivity.

The sensitivity experiments demonstrated that the ddPCR was one order of magnitude more sensitive than the qPCR. The limit of detection was 2.2 copy number (6 input copy number, 1:50 dilution of the positive sample), whilst the limit of detection for the qPCR was 36 copy number (30 input copy number, 1:10 dilution of the positive sample). When compared with the previously reported MLO detection techniques, the sensitivity in this study was higher when compared to PCR (0.1 pg/µL) (Lloyd et al., 2011; Cecchini et al., 2017). Other studies have also reported similar sensitivity results for ddPCR detection of *Flavobacterium psychrophilum* (1-240 gene copies/ml water; Lewin et al., 2020), spleen and kidney necrosis virus (1.5 copies/µL; Lin et al., 2020), and *Gyrodactylus salaris* parasite (7.8-8.8 copy/µL; Rusch et al., 2018).

Our ddPCR system showed a higher positive predictive value when compared to the qPCR, which indicated that ddPCR is a more effective method for bacteria detection, and showed a better reproducibility than the qPCR, both true especially when the bacterial DNA is at a low concentration (Lewin et al., 2020). All these features could be extremely useful in future RMS studies to investigate the presence of MLO in possible vectors such invertebrates and parasites (MLO seems to be able to be transiently and carried by *Ichthyophthirius multifiliis* – Pasqualetti et al., 2021*)*, water, eggs and environment in general where DNA can be scarce. Monitoring pathogen levels in water samples could aid early detection and surveillance especially in RAS systems and for research purposes.

The ddPCR data were direct measurements taken by a digital mechanism, without the need for standard curves (Cao et al., 2017). The digital system provided a total number of negative and positive droplets, which were used in the calculation of the absolute quantification by the Poisson distribution (Basu, 2017).

However, when compared to qPCR, the ddPCR exhibited a narrower range of detection because the ddPCR system generates approximately 20,000 droplets per reaction and counts the number of positive and negative droplets. When the template copy number is higher 20,000, the ddPCR droplets become completely saturated. This may be a limitation to the ddPCR method (Hindson et al., 2011), but it can be easily resolved by diluting the samples.

In summary, a new methodology for detection and absolute quantification of MLO has been established and applied in this study. The ddPCR method is repeatable and can detect extremely low concentrations of MLO DNA without the need for standard curves. This methodology might support further research on RMS by providing a powerful tool to detect the presence of MLO in the environment (through eDNA detection and quantification). Further work is needed to evaluate the repeatability and stability within and among laboratories.

In order to detect MLO in fish tissue samples with a high level of sensitivity, we established a ddPCR method for the detection and absolute quantification of MLO and compared the sensitivity and accuracy with qPCR. Our study has provided a powerful, accurate, sensitive and precise tool for the surveillance of RMS in fish samples and for use in general fish bacteria research.

# 6. Effect of temperature on experimental transfer of MLO (midchloridia-like organism) and development of red mark syndrome in rainbow trout

## 1. Introduction

As previously described, to date, diagnosis of RMS is based mainly on clinical, histopathological and molecular biology methods (Galeotti et al., 2021b; Orioles et al., 2022). In particular, histopathological features have been thoroughly described in literature, and different classifications have been reported (McCarthy et al., 2013; Galeotti et al., 2021b). MLOs have been visualized only in field cases through the use of transmission electron microscopy (Galeotti et al., 2017).

Studies of RMS so far almost entirely use diseased fish from farms, where the epidemiology and history of exposure to MLO is unknown (Metselaar et al., 2021). Thus, the fish has usually experienced a large range of temperatures during the incubation period, which typically lasts 600 degrees/days, as shown by cohabitation studies carried out at different institutions in Europe and UK (Verner-Jeffreys et al., 2008; Jørgensen et al., 2019, Metselaar et al., 2021). Since RMS cohabitation experiments have so far been conducted only at 12°C, disease progression at different temperatures needs to be formally established under controlled settings. This study aims to show if and how variation in the water temperature can influence pathogen transfer and disease development in a cohabitation model. Pathogen load in cohabitants is quantified by quantitative PCR and digital droplet PCR in spleen and skin, and pathology is evaluated by macroscopic and histological scoring of RMS lesions at different time-points. MLOs are visualized *in situ* by transmission electron microscopy on selected cases.

## 2. Material and Methods

### *2.1 Tank preparation*

Nine 180L cylindrical clear acrylic tanks were used for the trial; two cohabitation tanks and one negative control tank for each of the three temperatures. Flow-through water flowed into all tanks at 12ºC at a rate of approximately 25L/h. Temperatures of 15.5ºC and 19ºC were achieved by addition of aquarium heaters directly in the tanks (one in each on the 15.5ºC tanks and two in each of the 19ºC tanks). A few days before the start of the trial the tanks were filled with water and heaters installed and adjusted to reach the desired temperature in the tanks. The day before the experiment the heaters were shut off, allowing the water temperature to drop to 12±1ºC. Thus, on day 0 the fish were transferred from 12±1ºC to 12±1ºC and then slowly brought to the experimental temperature over 8 hours.

### *2.2 SPF and seeder fish preparation*

Specific pathogen free (SPF) fish were produced at the facilities at the host institution (DTU AQUA). These originated from eggs purchased from a commercial trout egg supplier (Aquasearch Ova, Billund, Denmark) certified free of the fish- pathogenic Infectious Pancreatic Necrosis (IPN), Infectious Hematopoietic Necrosis (IHN), Viral Hemorrhagic Septicemia (VHS) viruses, as well as the bacterium Renibacterium salmoninarum causing bacterial kidney disease (BKD), and reared at DTU AQUA in Lyngby (Denmark) according to Jørgensen et al. 2019. The eggs were disinfected upon arrival at the host institution and placed in hatching trays in the clean tank facilities at 8±1ºC, 12:12h light:dark cycle, commercial feed (BioMar, Brande, Denmark). After hatching they were slowly brought to 12±1ºC and transferred to 180L tanks where they were kept until used for experiments.

All fish were PIT (Passive integrated transponder)-tagged in the dorsolateral muscle just in front of the dorsal fin several weeks prior to experimentation so disease progression could be continuously followed in individual fish.

Seeders for the cohabitation experiment were produced by cohabitation of SPF fish with RMS-affected fish 55 days prior to start of the experiment. This type of seeder preparation model was already described by Jørgensen et al. in 2019 and Schmidt et al. in 2021 and since then the model has proved stable, with RMS symptoms developing consistently in more than 20 batches of fish. During seeder production fish were kept in 1000 L tanks in the infection facilities. Fresh conditioned facility water was supplied at a rate of ~100L/h and additionally an Eheim filter (model 2271 prof 4, Eheim, Deizisau, Germany) was mounted on the tank. Effluent water was pasteurized under pressure for at least 2min at 128°C and discharged into the sewer.

Disease transfer by cohabitation is only semi-standardized since individual seeders are infected and thus shed the pathogen at different levels. Thus, several seeders are used in each tank in order to increase the likelihood of obtaining comparable levels of pathogen load between tanks. Taking also the 3R principles into consideration we decided to use 3 seeders per tank, thus 18 seeders in total.

Balancing sampling regime and potential loss of fish with compliance with the 3R principles, 19 SPF fish were used as cohabitants per tank. This was the statistical minimum number of fish that could be used in the cohabitation model for it to be stable.

On Day 0 four seeder fish were cohabited with 19 SPF fish per tank, except for the negative control tanks to which only 19 SPF fish were added. The seeder fish were added blindly to each tank by associating a PIT code to each tank prior to cohabitation.

Temperature was then adapted as required by the experiment to 15.5°C and 19°C slowly over 8 hours.

Throughout the trial the fish were hand-fed 2mm pellets (BioMar, Brande, Denmark) at 1.0% body weight daily, except on the day prior to anaesthesia.

*2.4 Sampling*

The trial lasted 11 weeks during which fish and temperature were checked daily. At the day of starting the trial all fish were anesthetized in benzocaine (80 mg/L, Sigma-Aldrich, Brøndby, Denmark) after which they were weighed, measured and photographed.

At weeks 3, 8 and 11 weeks post cohabitation (wpc) all fish were treated as described above, and additionally macroscopic RMS pathology was scored for all fish. Samples were taken from 4 random cohabitants per tank (after euthanization in overdose of benzocaine). Samples consisted of: 1) Skin sample from an RMS lesion (or non-lesion site if no lesion was present) for MLO detection by qPCR; 2) skin scrape from a pre-determined standardized site for MLO detection by qPCR; 3) full-thickness (including muscle) lesion skin and control skin samples for descriptive histology. In case of multiple lesions, the lesion considered most severe macroscopically was sampled; 4) spleen for histological examination; 5) spleen for MLO detection by digital droplet PCR; 6) skin and spleen samples for MLO detection by Transmission Electronic Microscopy on selected cases (10 cases in total) with most evident and severe macroscopic lesions. On the day of termination of the trial all remaining fish were euthanized and sampled. In cases where there was pathology not obviously associated with RMS, swabs from affected sites as well as from kidney was plated on Tryptic Yeast extract Agar (TYA) and blood agar (BA).

*2.5 Macroscopic and microscopic lesion classification*

Tissues preserved in buffered formalin were processed for histology using standard protocols and 5 µm sections were stained with haematoxylin and eosin (H&E) and examined for the presence of RMS-associated pathology as described by Oidtmann et al. (2013) and using recent approaches proposed in literature (Orioles et al. 2019; Galeotti et al., 2021). RMS lesions were classified using

the macroscopic and microscopic lesion classification in Table 1 and Figure 1 into normal, mild, moderate, severe or healing/regenerative stages.

**Table 1:** Macroscopic and microscopic classification of RMS skin lesions. Modified from Orioles et al., 2019 and Galeotti et al., 2021,

| **Grade of severity** | **Macroscopic classification** | **Microscopic classification** |
|---|---|---|
| **Normal** | **Normal skin** | **Normal histology** |
| **Mild** | • Pale greyish or whitish with mild redness<br>• Mild exudate and swelling<br>• Visible scales | Prevalent lymphocytic inflammatory infiltrate can be absent to mild and limited to the dermis spongiosum or scale pockets in the initial stages; Evident scale pocket oedema is often observed;.<br>Epidermis is normal and other skin layers are mostly normal.<br>In more advanced stages can affect mildly the hypodermis and dermis compactum. Scales are mostly present and maintained. |
| **Moderate** | • From light to bright red<br>• Moderate exudate and swelling<br>• Mild scale loss | Hyperemia, congestion, some necrosis may be combined with a higher level of lymphocytic inflammation affecting dermis spongiosum and compactum and hypoderma.<br>The architecture differentiating dermis compactum and spongiosum is maintained. Dermis compactum could get thicker. Scale pockets are disappearing as replaced with inflammatory cells and oedema. Scales are often missing and osteoclasts/giant multinucleated cells are often observed |

| | | |
|---|---|---|
| | | reabsorbing the scales. Epidermis is thinner and can show exocytosis.<br>Muscle layer is generally not involved. |
| **Severe** | • Marked redness and hemorrhages<br>• Moderate to severe exudate and swelling<br>• Evident scale loss | Massive lymphocytic infiltration is seen in all dermis. There is no architectural differentiation between dermal layers.<br>Necrosis, congestion, haemorrhages often visible.<br>Epidermis is often affected showing signs of exocytosis and severe erosion/ulceration. Scales are often absent and scale pockets disappear.<br>Hypodermis is often heavily infiltrated and muscles can be variably affected |
| **Healing/regenerative stages** | • Lesions change to light brown color and progressively to grey<br>• Exudate and swelling disappears progressively<br>• Scales still missing in the initial stages but then regenerating | No or minimal lymphocytic inflammatory infiltrate with frequent presence of regenerative/young scales seen especially at the site of inflammation.<br>Otherwise normal or having characteristics similar to mild cases. |

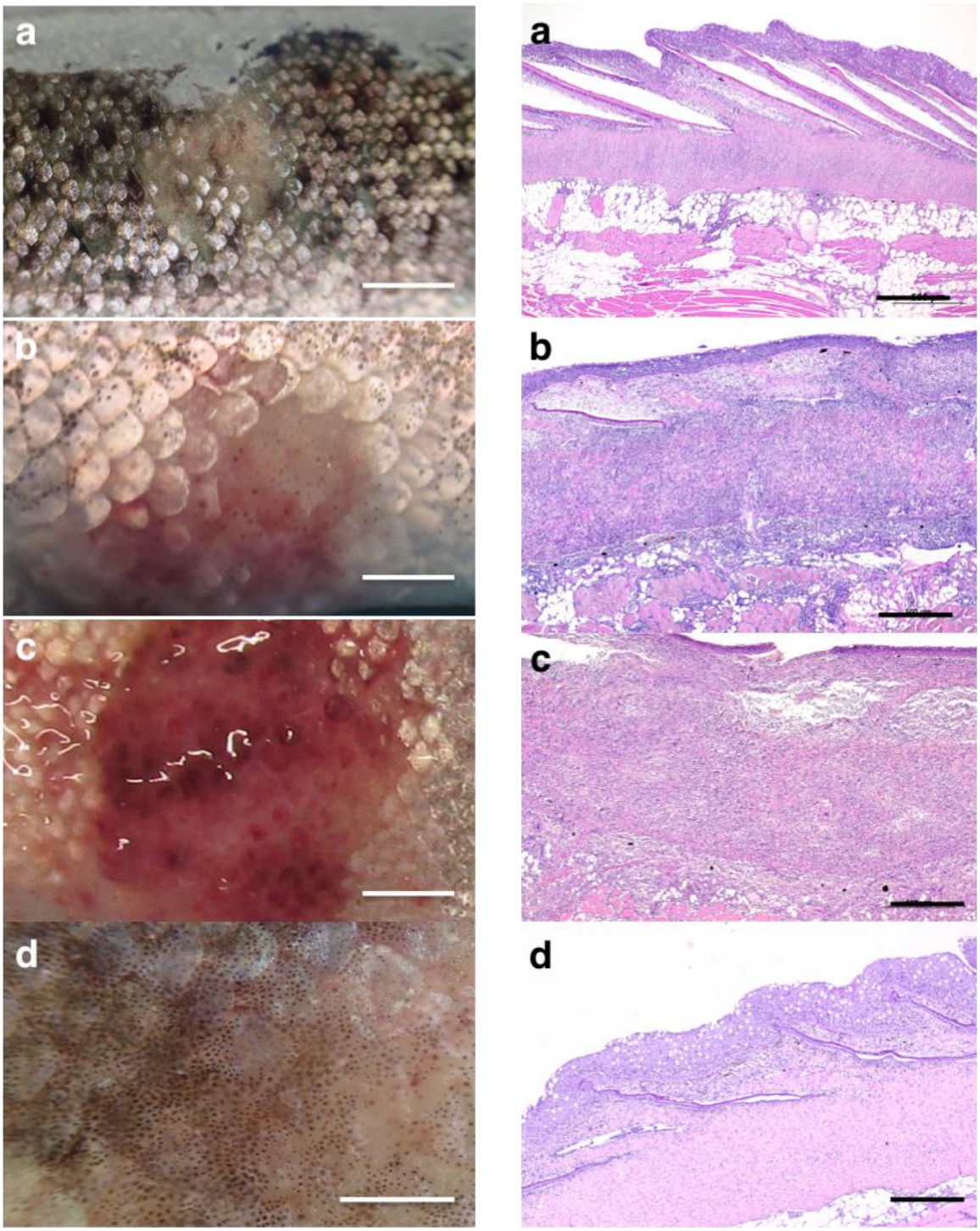



**Figure 1:** Macroscopic and microscopic examples mild (a), moderate (b) severe (c) and healing (d) RMS skin lesions (white bar=0.5cm, black bar= 500µm).

*2.6 Bacteriology and virology*

Selected cases were skin lesions or clinical signs not directly attributable to RMS were sampled for bacteriological and virological analysis.

TYA plates were incubated at 15°C and BA at 20°C until colonies formed. Selected bacterial colonies were picked and analysed with MALDI-TOF. The same fish sampled for bacteriology were also tested for virus by putting an organ pool homogenate on Bluegill Fry-2 (BF-2) and *Endothelial progenitor cells* (EPC) cultures following official diagnostic methods and procedures for the surveillance and confirmation of infection with VHSV and IHNV (https://www.eurl-fish-crustacean.eu/-/media/sites/eurl-fish-crustacean/fish/diagnostic-manuals/ihn-and-vhs/vhsv-and-ihnv-diagnostic-manual-v2021-2.pdf).

*2.7 DNA extraction, qPCR and ddPCR analysis*

Quantitative PCR analysis was performed on skin samples and skin scrapes.

Samples stored in RNA later were first transferred to a new sterile tube with 360µl ATL buffer.

DNA was extracted with the magnetic bead-based nucleic acid extractor IndiMag48 (Indical Bioscience, Germany) using the IndiMag Pathogen Kit (cat. No. SP947457, Qiagen for Indical Bioscience, Germany). Samples were pre-treated by bead-beating using one 5 mm stainless steel beads (Qiagen) for 2 min at 25 Hz (TissueLyser II, Qiagen) and then incubated with Proteinase K (40 µl) overnight at 56ºC with constant shaking. Then, samples were centrifuged at 14.000 rpm for 2 min and 100 µl of the supernatant was loaded on the robot (with 100 µl of sterile PBS). Then the extraction continued according to the manufacturer's instructions.

Subsequently, on the extracted DNA, MLO was detected with a SybrGreen-based qPCR (Brilliant II SYBR Green QPCR Master Mix, Agilent Technologies) as described in detail by Jørgensen et al. (2019), using MLO 16S rDNA primers, except that in the present analysis the use of plasmids was

omitted and quantification was thus not absolute, but instead relative to the internal calibrator insulin-like growth factor I (IGF-1). Samples were run in duplicate.

From the spleen samples (stored in RNA later) DNA was extracted using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions for tissues. Tissue was homogenized by bead-beating using one 5 mm stainless steel beads (Qiagen) for 2 times at 15 Hz per 20 sec, using Tissue Lyser II (Qiagen).

The ddPCR method was performed on the DNA extracted from spleen in accordance with the manufacturer's instructions (Bio-Rad, USA) and as described recently by Orioles et al., 2022.

*2.8 Transmission electron microscopy*

Samples were immersed for 4h in cooled 2.5% Glutaraldehyde, washed in Millonig's Buffer (0.1 M ph 7.4, 4 changes, 1h each) and stored in cooled buffer until the embedding.

Samples were immersed in 2.5% Glutaraldehyde (Agar Scientific, Cambridge Road Stansted Essex, UK) in PBS and stored at 4°C for 48 h. Subsequently, samples were washed in PBS (0.1 M, ph 7.2) and post-fixed with 1% Osmium tetroxide (OsO4) (Agar Scientific, Stansted, UK) in PBS for 1h. Samples were then washed with PBS and dehydrated in an ascending series of ethanol (Antonouli et al., 2020).

Following the immersion in propylene oxide (PO) for solvent substitution, samples were infiltrated in PO/Epoxy resin (1:1) overnight, embedded in the epoxy resin EMbed-812 (Electron Microscopy Sciences, 1560 Industry Road, Hatfield, PA, USA), for 48h at 60° C and sectioned using a Reichert–Jung Ultracut E ultramicrotome. Semithin sections (1 mm thick) were stained with Toluidine Blue, examined using LM (Zeiss Axioskop, Zeiss, Oberkochen, Germany) and photographed using a digital camera (Leica DFC230, Wetzlar, Germany). Ultrathin sections (60–80 nm) were cut with a diamond knife, mounted on copper grids, and contrasted with Uranyless and

lead citrate (SIC, Rome, Italy). They were examined and photographed using Philips TEM CM100 Electron Microscopes operating at 80 kV.

*2.9 Statistical analysis*

The statistical analyses were performed using R software version 4.0.4 (R Core Team, 2021). Normality of data distribution was tested using Shapiro-Wilk test. The effect of wpc (3, 8 and 11 weeks) and water temperature (wT°) (12 °C, 15.5 °C and 19°C) on MLO copy number and on qPCR data was assessed using aligned rank transform for nonparametric factorial ANOVA (Wobbrock et al., 2011) in ARtool package (Kay et al., 2021). Also, the interaction wT° × wpc was taken into account. Tukey test was considered for multiple comparisons. Fisher's exact test and post-hoc comparisons was used to test the association between micro and macro lesions and wT° or wpc. Spearman's correlation coefficient was used to assess the correlations among variables. Cohen's kappa coefficient was used to assess the agreement of MLO detection between qqPCR and qPCR. Probability of $P \leq 0.05$ was considered statistically significant.

*2.10 Ethical statement*

The experiment was conducted in agreement with animal experimentation permit number 2013−15−2934−00976 issued by The Animal Experiments Inspectorate of Denmark, and was additionally approved by the internal coordination committee for animal experiments at DTU. There are no in vitro alternatives to experimental animals for RMS, but number of animals was kept to the estimated necessary minimum. All individuals were PIT-tagged for individual recognition, thus allowing us to follow lesion development in individual fish and further reducing the number of experimental animals. All procedures were performed under anaesthesia.

## 3. Results

In total, 103 SPF fish were sacrificed and sampled at three time points at 3, 8 and 11 wpc. Necroscopy of internal organs was performed in all fish. Occasional (6 cases in total) splenomegaly was recorded, but in most fish no obvious internal pathology was observed.

### *3.1 Negative control fish*

All the SPF negative controls fish sampled at 3, 8 and 11 weeks of the trial for 12°C, 15.5° C and 19°C showed no signs of RMS-related skin changes and were all negative for MLO 16S rDNA in skin and spleen samples analysed.

### *3.2 Macroscopic and microscopic lesions development at different temperatures*

Sixty-five fish were sampled at 3, 8 and 11 wpc for macroscopic and microscopic evaluation of skin lesions in the cohabitation tanks at three different temperatures. These results are illustrated in figure 2 and 3 and reported in Table 2.

In total, macroscopically 34 lesions were normal, 7 mild, 3 moderate, 16 severe and 4 were in healing stages; microscopically 30 lesions were normal, 6 mild, 18 moderate, 5 severe and 5 healing. One fish skin sample was discarded due to poor quality. Examples of macroscopic and microscopic lesions are shown in figure 1.

Statistically significant associations were found between the severity of macroscopic lesions and wpc at 12 °C ($P<0.01$) and at 15.5 °C ($P<0.01$), but not with wpc at 19 °C ($P=0.65$).

Similar results were shown for microscopic lesions, statistically significant associations were found between severity of lesions and wpc at 12 °C ($P<0.01$) and at 15.5 °C ($P<0.01$), but not with wpc at 19 °C ($P=0.21$).

In particular, with regard to macroscopic lesions at 12 °C, the post hoc Fisher's exact tests revealed significant associations among all wpc ($P<0.01$). After 3 wpc all the fish (6) were normal (no lesions), after 8 wpc 5 fish had mild and 2 had moderate lesions, and after 11 wpc all fish had severe lesions. Very similar results were found for micro lesions, with the only exception that the lesions proportions were similar at 8 (1 and 6 fish with mild and moderate lesions, respectively) and 11 wpc (5 and 3 fish with moderate and severe lesions, respectively; $P\leq0.05$). Regarding macroscopic lesions recorded at 15.5 °C, the post hoc Fisher's exact tests revealed a significant association between 8 wpc and other sampling times ($P<0.01$), conversely, no association was found between 3 wpc (all fishes without lesions, 6) and 11 wpc (3 and 3 fishes without lesions and healing, respectively $P>0.05$). Similar results were observed for microscopic lesions. No associations between severity of lesion proportions and wpc was shown at 19 °C for both for macroscopic ($P<0.05$) and microscopic lesions ($P<0.05$) with most fishes that could be considered normal.

Significant associations were found between the severity of macroscopic lesion proportions and wT° after 8 wpc ($P\leq0.01$) and after 11 wpc ($P\leq0.01$), but not with wT° after 3 wpc ($P>0.05$). Similar results were found for microscopic lesions. Indeed, at 3 wpc macroscopic and microscopic skin of fishes was normal in all cases for all 3 temperatures except one case at 15.5 °C that showed signs of mild microscopic severity. At 8 wpc, the proportions of macroscopic lesion severity were different among wps. All (8) skin lesions at 15.5 °C were severe, at 19 °C most fishes were normal, in contrast at 12 °C, 5 and 2 fishes showed mild and moderate lesions, respectively. At 8 wpc, proportions of microscopic lesions severity were similar between 12 °C and 15.5 °C (most lesions were classified as moderate; $P>0.05$), in contrast, different proportions of microscopic lesion severity were observed at 19 °C (most fishes had not lesions). At 11 wpc, the proportions of macroscopic lesions and microscopic lesions severity were different at 12 °C than at 15.5 °C and 19 °C. In particular, at 12 °C all the fishes had severe macroscopic lesions, and 5 and 3 fishes had

moderate and severe microscopic lesions, respectively. Conversely, at 15.5 °C macroscopic and microscopic lesions were almost all normal or already healing, whereas at 19 °C most fishes had no macroscopic or microscopic lesions.

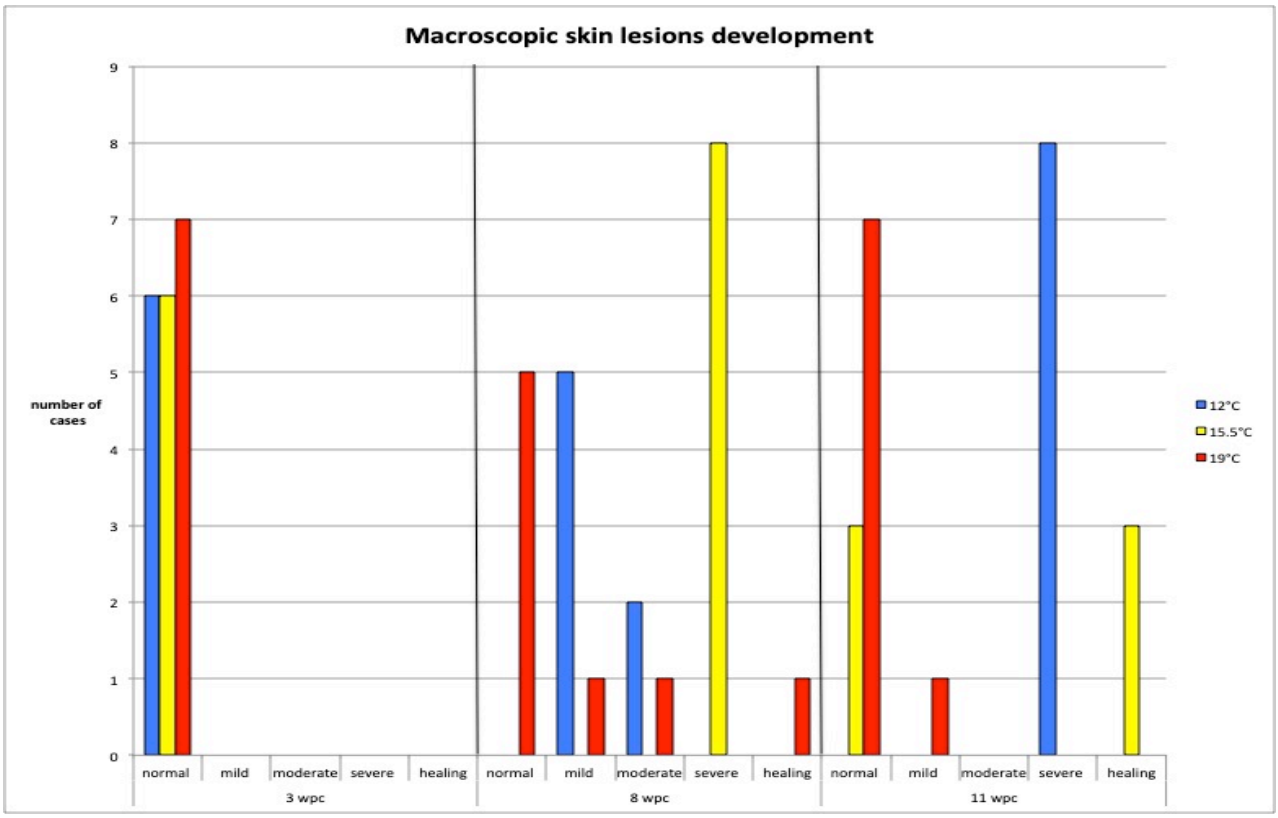



**Figure 2:** Macroscopic RMS skin lesions development at 3, 8 and 11 weeks post cohabitation (wpc) at 12° C (blue), 15.5°C (yellow) and 19° (red).

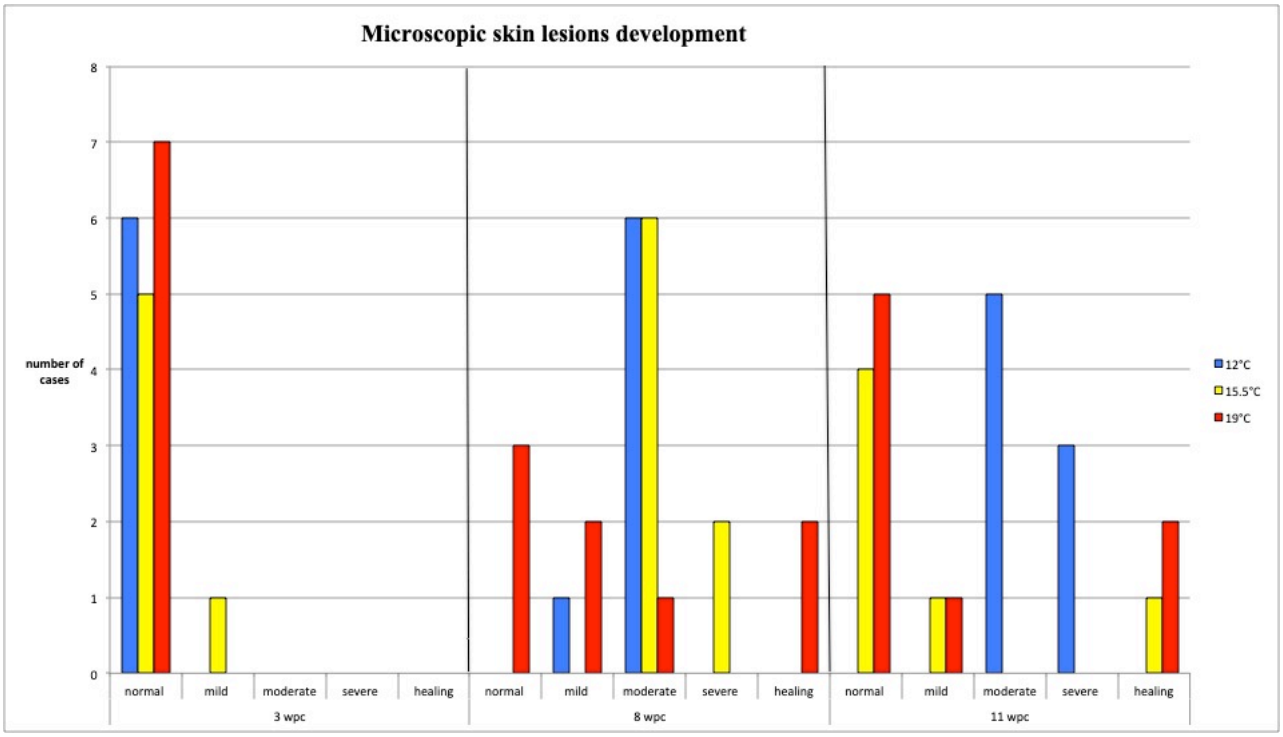



**Figure 3:** Microscopic RMS skin lesions development at 3, 8 and 11 weeks post cohabitation (wpc) at 12° C (blue), 15.5°C (yellow) and 19° (red).

**Table 2:** Cases sampled at 3, 8 and 11 weeks post cohabitation at three different temperatures and relative macroscopic, microscopic classifications and PCR results from skin and spleen samples.

| Case number | Time of sampling (weeks post cohabitation) | Temperature (°C) | Macro classification | Micro classification | DNA copies of MLO in spleen (ddPCR) | MLO Ct values in skin (qPCR) |
|---|---|---|---|---|---|---|
| 12 | 3 | 12 | normal | normal | 0 | no ct |
| 14 | | 12 | normal | normal | 0 | no ct |
| 15 | | 12 | normal | normal | 0 | no ct |
| 18 | | 15.5 | normal | normal | 0 | no ct |
| 19 | | 15.5 | normal | normal | 0 | no ct |
| 20 | | 15.5 | normal | normal | 0 | no ct |
| 22 | | 19 | normal | normal | 0 | no ct |
| 23 | | 19 | normal | normal | 0 | no ct |
| 25 | | 19 | normal | normal | 0 | no ct |
| 26 | | 19 | normal | normal | 0 | no ct |

| | | | | | | |
|---|---|---|---|---|---|---|
| 29 | | 19 | normal | normal | 0 | no ct |
| 30 | | 19 | normal | normal | 0 | no ct |
| 33 | | 19 | normal | normal | 0 | no ct |
| 35 | | 15.5 | normal | mild | 0 | no ct |
| 36 | | 15.5 | normal | normal | 0 | no ct |
| 38 | | 15.5 | normal | normal | 1,5 | no ct |
| 40 | | 12 | normal | normal | 2 | no ct |
| 43 | | 12 | normal | normal | 2 | no ct |
| 44 | | 12 | normal | normal | 0 | no ct |
| 46 | 8 | 19 | normal | normal | 0 | no ct |
| 47 | | 19 | normal | normal | 0 | no ct |
| 48 | | 19 | normal | mild | 0 | no ct |
| 49 | | 19 | mild | healing | 0 | no ct |
| 50 | | 19 | healing | healing | 0 | no ct |
| 51 | | 19 | normal | normal | 0 | no ct |
| 52 | | 19 | moderate | moderate | 0 | no ct |
| 53 | | 19 | normal | mild | 0 | no ct |
| 54 | | 15.5 | severe | severe | 4 | 34,28 |
| 55 | | 15.5 | severe | moderate | 17 | 30,64 |
| 56 | | 15.5 | severe | severe | 6 | 32,02 |
| 57 | | 15.5 | severe | moderate | 44 | 32,34 |
| 58 | | 15.5 | severe | moderate | 19 | 31,89 |
| 59 | | 15.5 | severe | moderate | 4 | no ct |
| 60 | | 15.5 | severe | moderate | 18 | 29,27 |
| 61 | | 15.5 | severe | moderate | 0 | 32,28 |
| 62 | | 12 | mild | moderate | 13 | 29,48 |
| 63 | | 12 | mild | mild | 0 | 32,33 |
| 64 | | 12 | moderate | moderate | 4 | 27,33 |
| 66 | | 12 | moderate | moderate | 10 | 27,81 |
| 67 | | 12 | mild | moderate | 12 | 31,23 |
| 68 | | 12 | mild | moderate | 14 | 31,71 |
| 69 | | 12 | mild | moderate | 8 | 29,81 |
| 82 | 11 | 12 | severe | severe | 10 | no ct |
| 83 | | 12 | severe | severe | 192 | 29,04 |
| 84 | | 12 | severe | moderate | 10 | 32,56 |
| 85 | | 12 | severe | moderate | 2 | 31,39 |
| 86 | | 12 | severe | moderate | 16 | no ct |
| 87 | | 12 | severe | moderate | 0 | 32,46 |
| 88 | | 12 | severe | moderate | 606 | 26,74 |
| 89 | | 12 | severe | severe | 0 | 32,15 |
| 90 | | 15.5 | normal | normal | 0 | no ct |
| 91 | | 15.5 | healing | mild | 0 | no ct |
| 92 | | 15.5 | healing | normal | 0 | no ct |
| 93 | | 15.5 | normal | normal | 2 | no ct |
| 94 | | 15.5 | healing | healing | 0 | no ct |
| 95 | | 15.5 | normal | normal | 7 | no ct |

| | | | | | | |
|---|---|---|---|---|---|---|
| 96 | | 19 | normal | healing | 0 | no ct |
| 97 | | 19 | normal | normal | 0 | no ct |
| 98 | | 19 | normal | normal | 0 | no ct |
| 99 | | 19 | mild | mild | 0 | no ct |
| 100 | | 19 | normal | normal | 0 | no ct |
| 101 | | 19 | normal | healing | 0 | no ct |
| 102 | | 19 | normal | normal | 0 | no ct |
| 103 | | 19 | normal | normal | 0 | no ct |

*3.3 MLO in skin and spleen samples*

Values of qPCR Ct ranged from 26.74 to 34.28; ddPCR value ranged from 1.5 to 606 DNA copies. Lowest qPCR Ct values (highest content of DNA) and highest ddPCR number of DNA copies were found in one of the most severe cases at 12°C sampled at 11 wpc, whereas no normal cases had detectable MLO DNA by qPCR in skin samples. In five cases with normal macroscopic and microscopic skin 1.5 to 7 copies of MLO DNA were detected by ddPCR.

The interaction water wpc × wT° was significant ($P<0.01$; data not reported in Tables) for both ddPCR and qPCR values. This means that the main effects, wpc and wT°, cannot be discussed independently. The interaction results for ddPCR and qPCR are reported in Table 3 and Figure 4, respectively. From the perspective of wpc: at 3 wpc very low levels of MLO was found and they were similar among wT° ($P>0.05$); at 8 wpc similar levels of MLO were found between 12 and 15.5 °C ($P>0.05$) while at 19 °C MLO was not found; finally, at 11 wpc the highest MLO levels were observed at 12 °C ($P\leq0.05$). From the perspective of wT°, at 12 °C, MLO values were lowest after 3 wpc ($P\leq0.05$) and similar values were found after 8 and 11 wpc ($P>0.05$). At 15.5 °C, the highest MLO presence was detected after 8 wpc ($P<0.05$), while at 19 °C, MLO was not found. Very similar results were found for qPCR values (Figure 4).

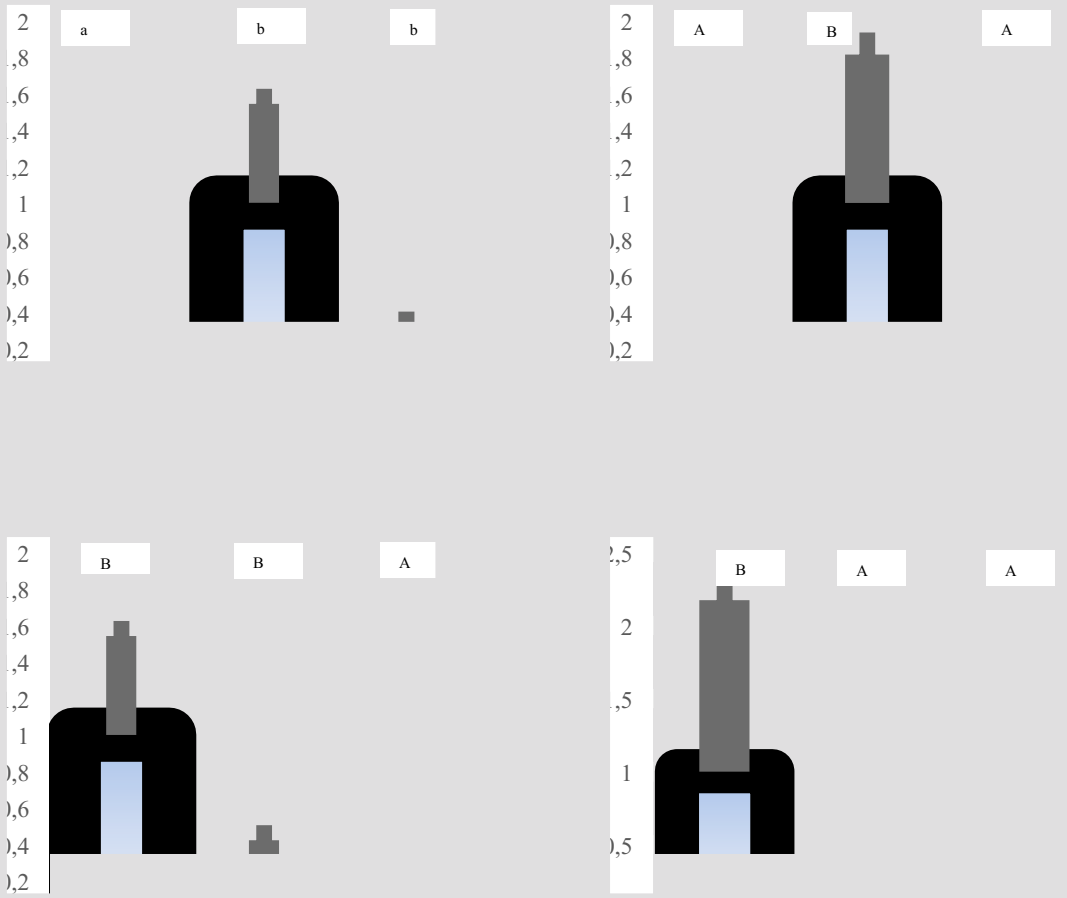
ddPCR and qPCR results had a percent agreement of about 81% and showed a moderate agreement in detecting MLO ($\kappa=0.59$; $P<0.01$; McHugh, 2012). Positive significant correlations were found

between ddPCR results and the severity of macroscopic (r=0.677; P<0.01) and microscopic (r=0.657; P<0.01) lesions without considering healing stages.

**Table 3:** Effect of water temperature and sampling time on the detection of *Midichloria*-like organism (MLO) DNA detected with ddPCR in spleen of fish exposed to RMS.

| | Water temperature (wT°), °C | | | RMSE | P-value |
|---|---|---|---|---|---|
| | 12 | 15.5 | 19 | | |
| MLO, DNA copy number | | | | | |
| 3 wpc | 0.0 [0.0-1.5]$^{e}$ | 0.0 [0.0-0.0]$^{e}$ | 0.0 [0.0-0.0] | 0.616 | 0.256 |
| 8 wpc | 10.0 [6.0-12.5]$^{Bf}$ | 11.5 [4.0-18.3]$^{Bf}$ | 0.0 [0.0-0.0]$^{A}$ | 8.255 | <0.001 |
| 11 wpc | 10.0 [1.5-60.0] $^{bf}$ | 0.0 [0.0-1.50]$^{ae}$ | 0.0 [0.0-0.0]$^{a}$ | 120.083 | 0.002 |
| RMSE | 122.93 | 8.519 | - | | |
| P-value | 0.030 | 0.002 | | | |

wpc. weeks post cohabitation; $^{a,b}$: Within the same row with unlike letters differ significantly at P≤0.05; $^{A,B}$: Within the same row with unlike letters differ significantly at P≤0.01; $^{e,f}$: Within the same column and variable with unlike letters differ significantly at P≤0.05. Data were expressed as medians [quartile 25% - 75% value]. RMSE= root mean square error.




ed according to the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001) and showed as fold change ratio (+se) with 8 week post cohabitation (wpc) or 12 °C water temperature (wT°) as reference; $^{A,B:}$ P<0.01; $^{a,b:}$ P<0.05.

*3.4 Bacteriology and virology*

Five fish where tested as describe above (i.e. on BA (20°C) and TYA (15°C)) due to the presence of small mouth ulcers and inflamed nostrils. Kidneys were plated as well as site of pathology (either small mouth ulcer (3) or inflamed nostrils (2)). Results showed pure culture of *Flavobacterium psychrophilum* from small mouth ulcer and nostrils at 3 wpc in 3 fish and mixed culture from renal

tissue.

At 8 weeks mixed culture from similar small lesions and one colony of *Flavobacterium psychrophilum* from kidney were detected.

All these fish were also tested for virus by putting an organ pool homogenate on cells (BF-2 and EPC) but no virus growth was detected.

*3.5 Electronic microscopy analysis*

In the selected samples from RMS-affected trout, ultrastructural observations indicated the presence of scattered microorganisms. These were mainly detected inside the cytoplasm of the cells. The bacteria were oval cells (400-800 x 100-200 nm) with an electron dense matrix and a lucid cell wall arranged as a trilaminar membrane (figure 5).

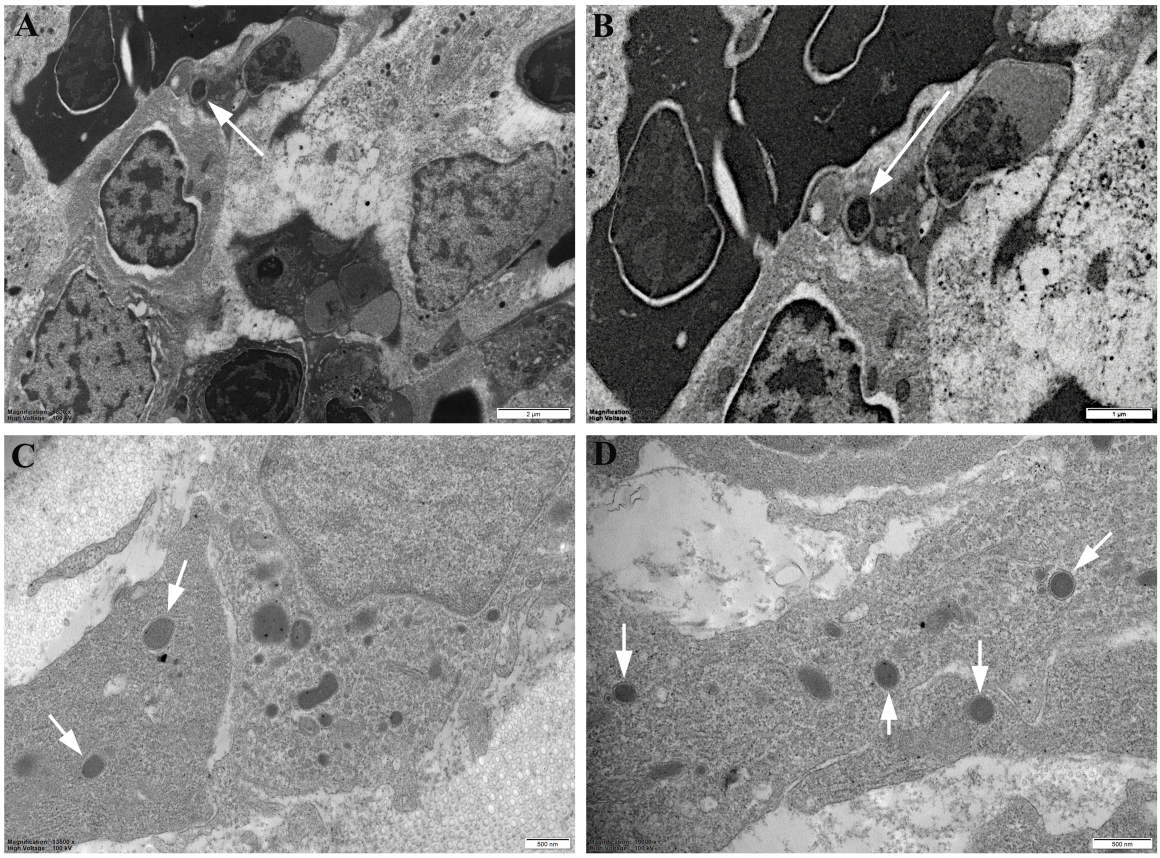



**Figure 5:** Representative micrograph of Spleen and Skin of RMS Rainbow Trout (Ultrathin section: 80 nm): A. Oval or round shaped microorganism, surrounded by a clear halo in the spleen tissue.

Magnification: 5800 X (Bar: 2µm). B. Round shaped microorganism, localized in the proximity of erythrocyte in the spleen tissue. Magnification: 10500 X (Bar: 1µm). C. Transmission electron micrograph of round shaped microorganisms, with evidence of trilaminar cell wall structure, in the skin. Magnification: 13500 X (Bar: 500 nm). D. Multiple round shaped microorganisms in RMS skin lesion. Magnification: 19000 (Bar: 500 nm). Arrow: microorganism.

*3.6 Confounding factors*

In the first two days of the study, some individuals in the 15.5°C and 19°C tanks got burn lesions due to contact with the aquarium heaters. In some (3 at 19°C and 1 at 15.5°C) fish the lesions were so severe that they were euthanized. In others, the lesions were less severe and only had a minor effect on appetite and behaviour. Arrangements were made to avoid direct contact between fish and heaters and there were subsequently no further problems with burns.

## 4. Discussion

The present study describes for the first time the development and resolution of RMS skin lesions at 15.5°C and 19°C in a well-established experimental setting. Furthermore, MLOs were visualized in selected cases through TEM, corroborating previous observations performed on field cases (Galeotti et al., 2017b). Even though no direct conclusions can be drawn from TEM results at this stage, the present study is a step closer towards establishing aetiology for RMS. Considering Friedricks and Relman's principles (Friedrick and Relman, 1996), where the supposition of a causative role could be supported by the association of higher numbers of a nucleic acid sequence and severity lesions, the results of this study provides strong evidences that MLO is the etiological agent of RMS. We described the association between the pathogen and severity of macroscopic and microscopic lesions and visualize MLO in the same associated samples; concentration of MLO

DNA detected by both qPCR and ddPCR techniques seem to follow the same trend of the severity of skin lesions at 12°C and 15.5°C. On other hand, no normal cases had detectable MLO DNA by qPCR in skin samples, providing further evidences for this candidate agent.

Ultrastructural observations detected single, electrodense, oval or round shaped microorganism, with a variable size and surrounded by a clear halo. As suggested by previous studies, these microorganisms were preferentially visualized in splenic erythrocytes/macrophages and skin macrophages/fibroblasts (Galeotti et al., 2017b): specifically, our TEM examinations detected the presence of this microorganism in the proximity of splenic erythrocytes and inside skin fibroblasts. In addition, the high resolution of electron microscopy (Giusti et al., 2019) allowed to visualize specific ultrastructural details of the microorganism that showed a cell wall composed by a clear outer and inner membrane (cellular envelope) indicating a trilaminar structure. According to previous studies, this ultrastructural feature is perfectly in line with the distinct trilaminar structure, frequently observed in Gram negative bacteria (Galeotti et al., 2017b).

Several macroscopical and microscopical classifications have been proposed in literature (Mccarthy et al., 2013; Orioles et al., 2019; Schmidt et al., 2020, Galeotti et al., 2021). Apart from healing stages, here the authors considered the most recent microscopic classification which was already statistically correlated with the progression of macroscopic lesions (Galeotti et al., 2021b). Healing stages were included as considered particularly important, especially at macroscopic level, to estimate the resolution of the disease when fish reach market size. However, correlation between microscopic and macroscopic healing stages requires further studies in order to be validated.

The results reported illustrates that RMS skin lesions can develop at 15.5°C and to some extent even at 19°C. However, at 15.5°C the development and resolution of lesions progress faster than at 12°C, and at 19°C lesions do not severely develop. The difference is mainly based on the severity of skin lesions and the rate of progression of the disease. If at 12°C the disease seems to be following a standard known kinetic with peak of severity of skin lesions observed at 11 wpc, at 15.5°C the disease seems to reach the peak earlier (at 8 weeks) and resolve almost completely at 11

weeks. RMS does develop only in mild forms and in few cases at 19°C with a peak of microscopic and macroscopic severity represented by one moderate case at 8 wpc. These results statistically explain the seasonal trend of RMS frequently observed and reported in literature (Ferguson et al., 2006; Oidtmann et al.,2013; Henriksen and Schmidt, 2017) and suggest that if it is possible to raise the water temperature, it is possible to accelerate the progression of the disease and to reduce the severity of pathology.

In this study, a specific digital droplet PCR previously designed for MLO (Orioles et al., 2022) has been applied successfully on experimental cases. The sensitivity of this relatively novel technique, detecting up to 1.5 copies of MLO DNA in spleen samples even in cases with apparently normal macroscopic and microscopic skin, is indeed very high, and can be certainly used in future studies to detect MLO's DNA in samples with low bacterial loads. The positive correlation of ddPCR values with macroscopic and microscopic lesion severity might be due to its higher sensitivity when compared with qPCR technique (Orioles et al., 2022). This technique may be used both as surveillance of possible transmission routes and potential sources in future studies.

In this study, ddPCR and qPCR showed a moderate agreement in detecting MLO ($\kappa$=0.59; $P<0.01$; McHugh, 2012); these two techniques were applied to the same fish but in different tissues, so complete agreement is not expected, and this cannot be considered a comparison of methods. The pathogenesis of RMS and possible MLO are to date not known, but spleen and skin lesions seem to be the most involved organs (Metselaar et al., 2021). Concentration of MLO DNA detected by both qPCR and ddPCR techniques seem to follow the same trend of the severity of skin lesions, at least at 12°C and 15.5°C.

It would be auspicable to use only the more sensitive ddPCR technique to evaluate MLO copies in different organs in order to further investigate the pathogenesis of RMS.

As previously described (Jørgensen et al. 2019 and Schmidt et al. 2020), we used cohabitation as the method of transferring RMS to naive fish in this study. Given the presence of *Flavobacterium*

*psychrophilum,* detected in small mouth and nostrils lesions, this cohabitation model has to be considered a co-infection model. Even though no bacteria were seen on histological analysis, however we cannot rule out that *F. psychrophilum* could have increased lesion severity.

Finally, the confounding factors due to burn lesions due to aquarium heaters were considered an incidental loss not significant from a statistical point of view.

To conclude, this study added more important details to the pathogenesis and development and diagnostics of RMS and for the first time illustrates through TEM the presence of MLOs associated with RMS in an experimental setting. The results described provide strong evidences that MLO is the etiological agent of RMS.

# 7. Observations from cases in recirculating aquaculture system

The outbreak under study occurred in a Slovenian rainbow trout recirculating aquaculture system (RAS) in March 2022 (water temperature 11-12°C) (Figure 1). Eggs came from a local domestic supplier. Fish were reared in concrete basins from 10 grams up to 150 grams before being transferred to another farm to reach commercial size. Rainbow trout were fed a standard commercial pelleted diet.

Water was taken from a local spring and no other fish farms were supplied from the same water source either upstream or downstream.

**Figure 1:** Slovenian rainbow trout recirculating aquaculture system (RAS).

About 50% of fish were showing signs compatible with RMS as shown in figure 2. No mortality was observed and affected fish had normal food intake and growth rate.

**Figure 2:** Typical RMS macroscopic skin lesions. Gross skin lesions ranging from 1 to 2 cm, were round to oval, reddish and covered by serous exudate associated to multifocal hemorrhages Lesions were focal (8/10) to multifocal (2/10) placed on ventral flanks and abdomen. Desquamation and skin ulcers were present in 3 out of the 10 trout. Bar =1cm.

Ten symptomatic fish were sampled and killed with an overdose of MS222 for investigation purposes. Fish's weight ranged from 35 to 90 grams and they were all in good body condition. Approximately 1 ml of blood was collected from the caudal vein and put in a 1.5 ml sterile plain

tube where it was allowed to clot to extract DNA. Samples of internal organs (gills, intestine, spleen, liver, kidney, heart) and samples from skin lesions and spleen were taken for histological and molecular biology analysis respectively. Tissues obtained for histological examination from skin/muscle and internal organs were fixed in buffered formaldehyde and then processed by an automatic histoprocessor (TISBE, Diapath, Italy) to be embedded in paraffin (ParaplastPlus, Diapath). Serial 5 μm sections were stained with haematoxylin–eosin.

QIAmp DNA Mini extraction Kit (Qiagen, Hilden, Germany) was used on skin and spleen samples and on blood clots, using Tissue Lyser II equipment (Qiagen, Hilden, Germany). The concentration and purity of the extracted DNA were assessed using a 'NanoDrop One' spectrophotometer (Thermo Scientific, Waltham, MA, USA) and the DNA was then sent to the digital droplet PCR (ddPCR) analysis.

The ddPCR method was performed on the DNA extracted in accordance with recent literature (Orioles et al., 2022a) and manufacturer's instructions (Bio-Rad, USA).

Twenty mls of water samples that were transferred into 50 mL centrifuge tubes containing 1.5 mL of sodium acetate (3 M) and 28.5 mL of absolute ethanol for DNA preservation (modified from Gomes et al., 2017). Four 20mls of distilled water transported from our laboratory (Udine University) as negative control samples were taken. A direct DNA precipitation and extraction method was adopted for water, and 50 ml tubes containing water and preservative were centrifuged at 3200 x g for 60 min at 6 °C. The resulting supernatant was discarded, a part approximately 300 μl, that was used to suspend the cells pellet. The suspension was transferred to a 1.5 ml centrifuge tube and centrifuged at 5000 x g for 10 min. DNA was extracted from the cells pellet by QIAmp DNA Mini Kit Qiagen, Hilden, Germany) following the bacterial pellet protocol.

Gross skin lesions ranging from 1 to 2 cm, were round to oval, reddish and covered by serous exudate associated to multifocal hemorrhages Lesions were focal (8/10) to multifocal (2/10) placed on ventral flanks and abdomen. Desquamation and skin ulcers were present in 3 out of the 10 trout.

Necroscopy revealed splenomegaly in two fish. No abnormalities were detectable in the other organs. Cytology of skin and gills did not reveal the presence of parasites.

Histological examination of skin samples revealed a marked lymphocytic and macrophagic inflammatory infiltration involving all skin layers, including the hypodermis and muscular tissue (Figure 3); the infiltration of lymphocytes and macrophages in the stratum spongiosum of the derma was moderate to severe with frequent scale pockets oedema and cellular infiltration; frequent scale reabsorption and thickening of the stratum compactum of the derma, infiltrated by lymphocytes and macrophages was observed in most severe cases (Figure 3). The underlying muscular tissue was also affected by lymphocytic and macrophagic cellular infiltration in moderate and severe cases. The two macroscopically abnormal spleens showed severe sinusoids congestion. No evidence of bacteria was observed. Histological examination of remaining organs did not reveal significant abnormalities.

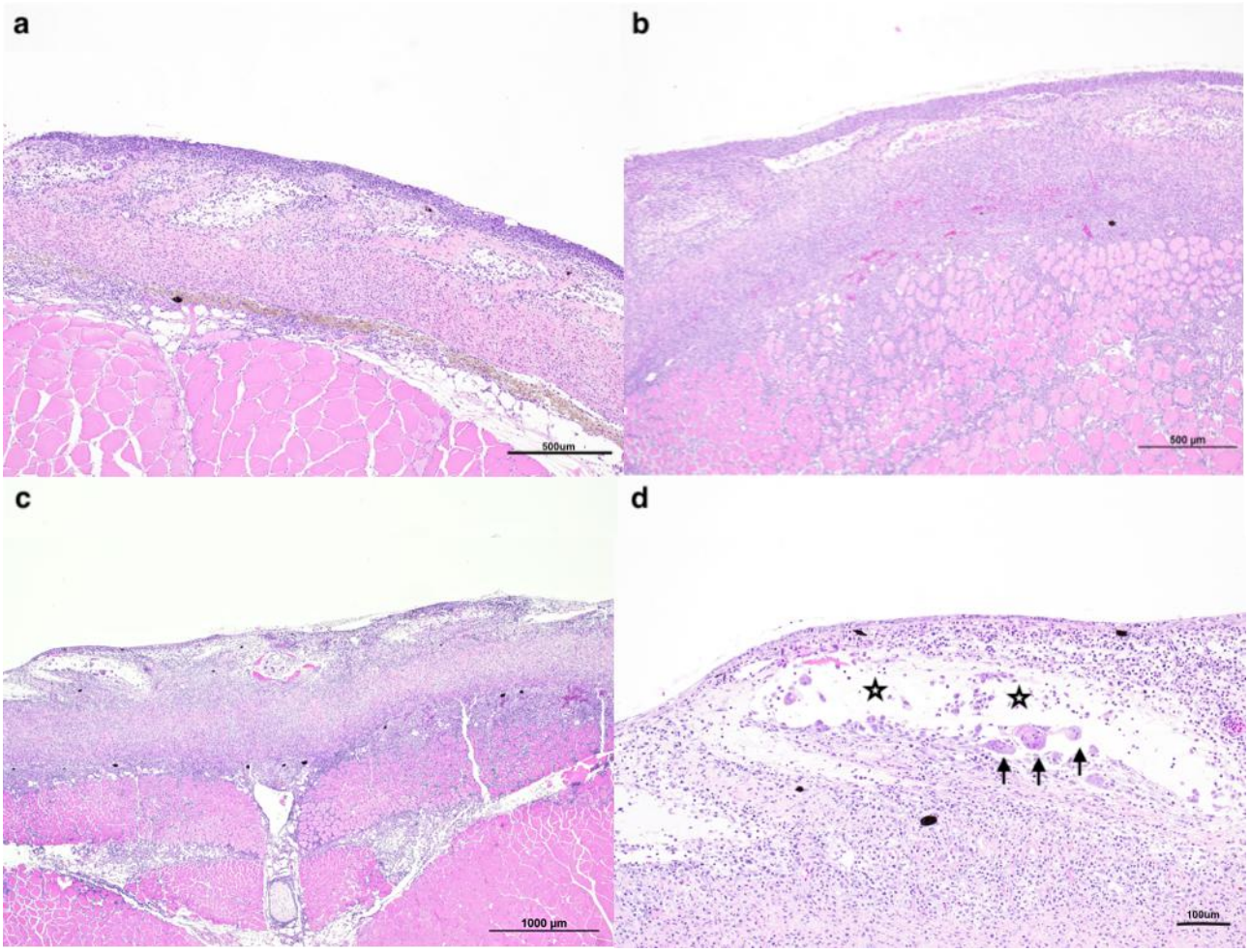


**Figure 3 (a-d):** H&E sections. Histological features of RMS skin lesion. In early moderate cases (a) infiltration of the lymphocytic and macrophagic inflammatory cells involve predominantly dermis spongiosum and hypoderma; the same type of inflammatory infiltration involves all skin layers with progressive infiltration and thickening of dermis compactum, hypodermis and muscles in moderate and severe cases (b and c). Scale pocket (black stars) oedema, inflammatory infiltration and scale reabsorption by giant multinucleated cells (black arrows) are frequently observed (d).

Skin lesions were classified as moderate or severe as reported recently in literature (Orioles et al., 2022b). Results of the molecular biology analysis are reported in Table 1.

**Table 1:** Digital droplet PCR detection of MLO DNA from skin, spleen, blood and water sample.

| Case n° | MLO DNA copies in skin (ddPCR) | MLO DNA copies in spleen (ddPCR) | MLO DNA copies in blood (ddPCR) |
|---|---|---|---|
| 1 | 250 | 7.5 | negative |
| 2 | 4300 | 2900 | 10 |
| 3 | 6560 | 4200 | 22 |
| 4 | 1560 | 324 | negative |
| 5 | 92 | 628 | 3 |
| 6 | 1700 | 410 | negative |
| 7 | 344 | 74 | 5 |
| 8 | 9500 | 4340 | 38 |
| 9 | 1000 | 334 | 4 |
| 10 | 54 | 16 | negative |

| Water samples | MLO DNA copies |
|---|---|

| | (ddPCR) |
|---|---|
| **Sample from RAS farm** | 40 |

Considering reported criteria for skin disorders in rainbow trout (Oidtmann et al., 2013) and the findings reported in this study, the skin lesions were consistent with RMS. This is the first report of RMS affecting rainbow trout in a RAS system and the evidence that RMS could affect rainbow trout under 50 grams (smallest was 35grams) of weight, developing severe lesions similar to what occurs in market size fish. The manuscript by Oh et al. (2019) describes several cases of the disease in rainbow trout in South Korea, in which macroscopic lesions resembling RMS can be seen in juvenile rainbow trout weighed less than 30 g. The high level of mortality observed by Oh et al. (2019) does not reflect the classic case definition for RMS though and further investigations were suggested as necessary to confirm the RMS status of these fish (Metselaar et al., 2022).

Furthermore this study represents the first successful attempt to detect MLO DNA within water.

Even though final confirmation would require further analysis, in this case eggs and lack of biosecurity measures might have been the possible sources of infection. The results confirm ddPCR as a very sensitive method to assess MLO DNA quantitatively; blood may be an appropriate matrix where to detect MLO and might be used for culturing this organism as MLO may have a bacteriemic phase, in its strictest sense, and distributes in different organs. However, the pathogenesis of RMS and the potential role of MLO have yet to be elucidated.

# Conclusion and future perspectives

In this PhD project we successfully established a strong association between MLO and the development of RMS. This was possible considering Friedricks and Relman's principles (Friedrick and Relman, 1996), useful in cases of uncultivable microorganisms, where the supposition of a causative role could be supported by the association of higher numbers of a nucleic acid sequence and severity of lesions. We proposed an histological scoring sytem, including healing stages, which was positively correlated with qPCR values for MLO DNA. To further support these evidences, MLO was visualized for the first time in experimental conditions in RMS affected tissues by using transmission electron microscopy.

Furthermore, RMS development at different temperatures was formally established. RMS skin lesions can develop at 15.5°C and to some extent even at 19°C. However, at 15.5°C the development and resolution of lesions progress faster than at 12°C, and at 19°C lesions do not severely develop. The difference is mainly based on the severity of skin lesions and the rate of progression of the disease.

Experimentally infected spleen and skin samples where used to develop a novel molecular technique with superior sensitivity for MLO DNA. This technique was applied to field cases where MLO DNA was detected for the first time from water and blood samples. RMS was then described in a RAS system and in juvenile trouts up to 35 grams.

RMS literature was critically revised to find the gaps in current research and future perspectives.

A structured survey study was designed to provide an epidemiological picture of the distribution of the virus in Italian rainbow trout farms and we described this disease in a new country, Bosnia-Herzegovina. This has provided more evidences that the disease is still a major concern for trout aquaculture sector and highlighted the lack of studies on disease-associated costs.

Finally yet importantly, a solid network of collaborations, including private companies and institutions around Europe, was created to support this project and future studies.

In order to achieve significant progress on the characterization of this disease, future perspectives should:

1) Establish the genome of the MLO associated with RMS or cultivate the MLO in experimental conditions. In addition to 16S region analysis, previously explored for MLOs, other genomic regions (htrA, gltA, Sca4, ompA, and ompB genes) used for molecular characterization of Rickettsiales could be assessed to detect and characterize bacteria found in skin lesions. The identification of the causative agent could be carried out by isolation and characterization of bacteria consistent with MLOs from rainbow trout samples or by molecular biology and/or nanotechnology methods aimed at the detection of not-culturable microorganisms. Such approach could involve traditional (isolation on cell cultures) and biomolecular (PCR, qPCR, DNA hybridization based, biosensor) techniques. In order to enhance the possibility to isolate MLOs, a number of continuous immortalized cell lines (SSE-5, CSE-119, SHK-1, RT gills) could be tested.
2) Study Rainbow trout immune response to RMS and its pathogenesis. The immune response gene expression profile could consider pro-inflammatory cytokines (IL-1,IL-10, TNF- a, IL-8, IL-6); 2) innate immunity (TLR2, TLR4, TLR5), acute phase reaction (C3-F, SAA-F), macrophage activation (M-CSFR-F) and adaptive immunity (IgT, IgM, MHC-Ib antigen, MCH-II, TCR-). Immune response may be also evaluated by the application of specific immunohistochemical biomarkers in spleen, kidney and skin specimens of infected RT. Previous observations of in naturally infected fish pointed out a marked splenomegaly in RT with RMS suggesting a systemic involvement and the need of further investigation to elucidate the infection pathway, including MLOs way of entry and processing in hemo-lymphopoietic organs.

3) Use ddPCR technique developed in this project to both assess the presence of MLO DNA in the environment and originate predicting indexes of the outbreak of the disease. This could lead to implement biosecurity measure and help the farmers to anticipate the development of RMS lesions.

   The role of invertebrates and parasites, as already explored for *Ichthyophthirius multifiliis*, a holotrichous protozoan recently associated with MLOs infection in trout, should be investigated further by using ddPCR.

4) Study in details the potential impact of this disease on production cost and find viable commercial alternatives to those farmers, which seems more affected by RMS, like organic trout farmers and portion table trout producers.

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