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A novel DNA-based *in situ* hybridization method to detect *Desmozoon lepeophtherii* in Atlantic salmon tissues

Running title: *In situ* hybridization to detect *Desmozoon lepeophtherii*

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DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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CONFLICT OF INTEREST

The authors declare that there are no potential sources of conflict of interest with this work.

ETHICAL APPROVAL

NA

ABSTRACT

The microsporidian *Desmozoon lepeophtherii* Freeman and Sommerville, 2009 is considered significant in the pathogenesis of gill disease in Atlantic salmon (*Salmo salar* Linnaeus, 1758). Due to the difficulty in detecting *D. lepeophtherii* in tissue sections, infections are normally diagnosed by molecular methods, routine haematoxylin and eosin (H&E) stained gill tissue sections and the use of other histochemical stains and labels to confirm the presence of spores. An *in situ* hybridization (ISH) protocol specific for *D. lepeophtherii* was developed using DIG-labelled oligonucleotide probes. Diseased Atlantic salmon gills, were analysed by ISH, calcofluor white (CW) and H&E. All methods showed high levels of specificity (100%) in their ability to detect *D. lepeophtherii*, but the sensitivity was higher with ISH (92%), compared with CW (64%) and presence of microvesicles on H&E stained sections (52%). High levels of *D. lepeophtherii* spores were significantly associated ($p < 0.05$) with the development of *D. lepeophtherii*-associated pathology in the gills, with Ct values below 19 and over 100 microsporidia/10 mm^2 of gill tissue (from the ISH counts) seemingly necessary for the development of microvesicles. The ISH method has the advantage over other histological techniques in that it allows all life-stages of the microsporidian to be detected in infected salmon gill tissue sections.

KEYWORDS: *Desmozoon lepeophtherii*; *Paranucleospora theridion;* microsporidian, gill disease; ISH

1. INTRODUCTION

The microsporidian *Desmozoon lepeophtherii* Freeman and Sommerville, 2009 (syn. *Paranucleospora theridion*) is one of the most prevalent agents detected by polymerase chain reaction (PCR)-based assays in the gills of marine farmed Atlantic salmon (*Salmo salar* Linnaeus, 1758) in Europe, irrespective of the health status of the fish (Downes et al., 2018; Gjessing et al., 2019; Steinum et al., 2010). Higher burdens of *D. lepeophtherii* have been observed in the gills of Atlantic salmon during the autumn months (Gunnarsson et al., 2017), the season in which outbreaks of gill disease associated with *D. lepeophtherii* are usually reported (Matthews et al., 2013; Weli et al., 2017). From gross examination, gills affected by *D. lepeophtherii* present with areas of paleness and swollen filaments (pers. obs.). Histologically, gill lesions associated with the microsporidian include necrosis, hypertrophy and hyperplasia of the lamellar epithelial cells, infiltration by phagocytic cells and cell debris with associated pigmented material (Matthews et al., 2013; Gjessing et al., 2019). However, the pathology changes through the course of the disease. Matthews et al. (2013) described a *D. lepeophtherii* infection in farmed Atlantic salmon in Scotland with findings including lamellar epithelial cell proliferation, infiltration by inflammatory cells and the presence of necrotic and hypertrophied gill epithelial cells in association with the presence of microsporidian spores. Fish sampled one week later had less severe inflammatory cell infiltrate and necrotic lesions and fewer *D. lepeophtherii* spores present in the gills but prominent lamellar epithelium hyperplasia and hypertrophy was still present. Weli et al. (2017) reported *D. lepeophtherii* to be the main cause for the clinical disease observed in gills during a longitudinal study of Norwegian farmed Atlantic salmon. Necrotic lesions were more severe during the acute stage of the disease (during early sampling time points), while chronic pathology (present during later sampling time points) was characterised by a

marked host response, including severe inflammatory cell infiltration and hyperplasia of the gill epithelium.

The life cycle of *D. lepeophtherii* is complex: one type of sporogony occurs in the sea lice (*Lepeophtheirus salmonis* Kroyer, 1837) and two different presumptive developmental cycles occur in salmon (Nylund et al., 2010). In salmon two different types of spores develop (Nylund et al., 2010): (1) auto-infective ~ 1 µm-diameter oval/spherical intracytoplasmic spores and (2) environmental ~ 2.5 µm-long x 2.0 µm-wide ellipsoidal intranuclear spores. Matthews et al. (2013) improved the sensitivity of detecting the small *D. lepeophtherii* auto-infective spores in salmon gills by using the Gram Twort stain. However, the technique severely under-estimates the total number of spores compared to other methods, such as staining with calcofluor-white (CW) or labelling by immunohistochemistry (IHC) (Herrero et al., 2020). Additionally, none of these methods facilitates the detection of the other pre-sporogonic stages of *D. lepeophtherii* which can be present. An *in situ* hybridisation (ISH) method was developed to detect *D. lepeophtherii* based on a large plasmid-encoded RNA probe (Weli et al., 2017). However, this method has practical difficulties due to lack of reproducibility in generating the probe, balancing the preservation of tissue morphology against adequate probe permeability and the labile nature of the RNA probe (Corthell, 2014).

In outbreaks of complex gill disease, which is caused by multiple aetiological agents (Herrero et al., 2018), the lesions caused by each individual agent can be difficult to differentiate, including those caused by *D. lepeophtherii*. Additionally, the high prevalence of the microsporidian in salmon populations, the rapid change in the progression of the pathology and the difficulty of detecting the parasite with histochemical stains make the study of gill disease associated with this organism challenging. A more sensitive and specific method capable of detecting all stages of the parasite's life cycle, not just the

spores, would allow accurate detection of *D. lepeophtherii* and help gain insight into its specific histological lesions. *In situ* hybridisation has been used to successfully detect and visualise pre-sporogonic and sporogonic stages of the microsporidia *Enterospora nucleophila* Palenzuela et al., 2014 and *Nucleospora salmonis* Chilmonczyk et al., 1991 using probes against the small subunit ribosomal RNA (SSU rRNA) or intergenic regions of the rRNA, respectively (Ahmed et al., 2019; Grésoviac et al., 2007). Probes were applied to formalin fixed, paraffin-wax embedded (FFPE) tissue samples and resulted in more sensitive detection of the parasite compared with histochemical stains. A RNAScope® ISH protocol for the detection of multiple different targets, including *D. lepeophtherii*, was also used in a recent investigation of bacterial, viral and parasitic agents in complex gill disease of Atlantic salmon (Gjessing et al., 2021). This procedure, however, used proprietary probes whose specificity is difficult to assess.

The aim of this study was to develop and optimise an ISH protocol using DNA oligonucleotide probes for the species-specific detection of all life-stages of *D. lepeophtherii* in tissue sections which could be practical for research and diagnostic purposes. The detection of the parasite in infected tissue sections and associated pathology were compared with other histological staining methods, the results of which were correlated with *D. lepeophtherii* specific real-time reverse transcriptase PCR (RT-rtPCR).

2. MATERIALS AND METHODS

2.1 Samples used in the study

Archived marine stage Atlantic salmon gill tissue samples (n=28) obtained from farms located on the west coast of Scotland and collected between 2016 and 2017 were provided by PHARMAQ Analytiq (Inverness, Scotland). Tissue sections from fish with different burdens of *D. lepeophtherii* were selected for this study. All samples had been analysed

previously by RT-rtPCR, performed as described by Nylund et al. (2010), to quantify the relative amounts of *D. lepeophtherii*-specific RNA in gill samples, expressed as Ct values and presented in Supplementary Table 1. Samples positive for *D. lepeophtherii* by RT-rtPCR were used as positive controls and gill tissues confirmed as *D. lepeophtherii*-free by RT-rtPCR were used as negative controls to develop and validate the ISH protocol.

To test the specificity of the oligoprobes, tissue sections containing two closely related species of microsporidia from the Enterocytozoonidae family, *Enterocytozoon hepatopenaei* Tourtip et al., 2019 in the hepatopancreas of the black tiger shrimp (*Penaeus monodon* Fabricius, 1798) and *N. cyclopteri* Freeman et al., 2013 in the kidney of the lumpfish (*Cyclopterus lumpus* Linnaeus, 1758), were subjected to the ISH method.

Additional Atlantic salmon tissue sections used to optimise the ISH protocol included heart, liver, stomach, pancreas, spleen, intestine, pyloric caeca, kidney, muscle and skin. Other samples subjected to the ISH protocol included; a sea louse (*L. salmonis*) from a salmon farm in Scotland that had xenomas under its cuticle suggestive of *D. lepeophtherii* infection and gill tissue from farmed Atlantic salmon in Canada infected with *D. lepeophtherii* that was positive by specific RT-rtPCR (kindly provided by Prof S. Jones, Pacific Biological Station, Nanaimo, Canada).

2.2 Development of a DNA oligoprobe ISH protocol to detect *D. lepeophtherii*

Oligonucleotide probes specific for *D. lepeophtherii* were designed by aligning all the sequences of the microsporidian and phylogenetically close species available within the SILVA database release 132 (Pruesse et al., 2007, www.arb-silva.de). The dataset was mostly comprised of the SSU rRNA region, but some segments from the internal transcribed spacer (ITS) and partial large subunit (LSU) regions were also inspected. The alignment showed no significant sequence variations between genotypes from farmed

Atlantic salmon in Scotland or Norway, different species of sea lice (*Caligus elongatus* Nordmann, 1832 and *L. salmonis*), ballan wrasse (*Labrus bergylta* Ascanius, 1767) or rainbow trout (*Oncorhynchus mykiss* Walbaum, *1792*). Variation was observed between the Canadian and European genotypes of *D. lepeophtherii*, as reported previously by Jones et al. (2012). The alignment was pruned to the longest representative genotypes available for *D. lepeophtherii* and closely related Enterocytozoonidae microsporidia (Table 1). Regions showing sufficient variability between the genotypes of *D. lepeophtheri* and the closely related species were chosen to design appropriate antisense oligonucleotide probes. *In silico* analyses of the thermodynamic profiles and specificity were aided by the software package OLIGO 7 (Rychlik, 2007) and by NCBI BLAST search tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The five *D. lepeophtherii*-specific antisense oligonucleotide probes targeting the regions SSU rRNA (n = 3) and ITS (n = 2) designed for use in this study are presented in Table 2. Oligoprobes were synthesised and labelled with digoxigenin deoxyuridine triphosphate (DIG-dUTP) at the 5' and 3' ends of the probes (Eurofins Genomics, Ebersberg, Germany).

The ISH protocol was based on the method described by Palenzuela and Bartholomew (2002), with modifications. Formalin fixed, paraffin-wax embedded gill tissue samples were sectioned (4 µm), mounted on Superfrost plus coated slides (Menzel-Gläser, Braunschweig, Germany) and incubated at 60°C for 1 h. Sections were dewaxed in xylene, rehydrated through a decreasing ethanol concentration (100%, 95% and 70%) and then equilibrated in Tris-$CaCl_2$ buffer (200mM Tris, 2mM CaCl2, pH 7.2) for 10 min. As *D. lepeophtherii* is an intracellular pathogen, permeabilisation of the tissue was performed to allow intracellular penetration of the probes to improve binding to their target sequence. To optimise this procedure, serial sections were permeabilised with proteinase K (PK) (Roche, Welwyn, UK), 15 µg mL^{-1} in Tris-$CaCl_2$, for 10 and 30 min at 37°C.

198 Tissues not exposed to PK were also examined. Proteolysis was stopped with two washes
199 in 2x saline-sodium citrate buffer (SSC) (0.3 M NaCl buffer, 0.03 M trisodium citrate, pH
200 7.0) for 10 min each. Slides were covered with 400 µL of freshly made hybridization
201 mixture consisting of 112 µL nuclease-free water, 40 µL of 20x SSC buffer (3.0 M NaCl
202 buffer, 0.3 M trisodium citrate, pH 7.0), 100 µL of deionized formamide (Sigma-Aldrich),
203 8 µl Denhardt's solution, 80 µl dextran sulphate (50%, w/v) (Sigma-Aldrich), 40 µL of
204 10x PBS and 20 µL of DNA from fish sperm (MB- grade, Sigma-Aldrich). DIG-labelled
205 oligoprobe concentrations were adjusted to 100mM with Tris-EDTA buffer (TE). Equal
206 concentrations of different probes were mixed together to make two cocktails. Cocktail 1
207 (C1) comprised probes 16L21, 819L25 and 1339L25. Cocktail 2 (C2) comprised probes
208 1284L21, 1002L25 and 1339L25. Both cocktails were prepared at various dilutions
209 (1/1000, 1/500 and 1/200) in hybridization buffer. Each oligonucleotide probe (1/1000
210 dilution) was also tested individually on serial sections. Slides with the probes applied
211 were placed on a heating block at 95ºC for 10 min. After two min at this temperature,
212 slides were covered with Hybri-slips (Sigma-Aldrich) and incubated at 37˚C overnight to
213 hybridize to complementary sequences. After overnight incubation, slides were rinsed
214 with 2x SSC buffer to remove the Hybri-slip. Stringency washes were then performed,
215 using 2x 10 min washes in 2x SSC buffer, to remove any unbound probes and assessed at
216 two different temperatures, 37°C and 45°C, 2x 10 min in 1x SSC buffer at 37°C and,
217 finally, once in 0.25x SSC buffer for 10 min at 37°C for C1. The same wash procedure
218 was used for slides incubated with C2, except for the last stringency wash which was
219 performed with 0.5x SSC buffer for 10 min, again assessing at two different temperatures,
220 37°C and 45°C. The slides were shaken slowly during each wash. Following the stringency
221 washes, the tissue sections were transferred to wash buffer A (1 M Tris base, 1.5 M NaCl,
222 pH 7.5) for 10 min at 24˚C. Subsequent to this, sections were incubated for 1 h at 21°C

with blocking solution (2% sheep serum, 0.1% Triton X-100 in wash buffer A) to prevent non-specific antibody binding. Bound probes were detected by covering the slides with a commercial sheep anti-DIG antibody (Fab fragments) conjugated to alkaline phosphatase (AP) (Roche, Manheim, Germany, reference 11093274910) diluted 1:200 in blocking solution for 2h at 21°C. Unbound antibody was removed using wash buffer A for 2 x 10 min and then incubating in wash Buffer B (100 mM Tris, 100 mM, NaCl, 50 mM, MgCl2, pH 9.5) for 10 min, with agitation, at 21°C. The reaction development was optimised by incubating the slides for either 3 h or overnight with AP enzyme substrate NBT/ BCIP (5-bromo-4-chloro-3-indolyl phosphate and 4-nitro-blue tetrazolium chloride) (Roche), diluted 1:500 in wash Buffer B, without $MgCl_2$, at 21°C in the dark. The visualisation reaction was terminated by placing the sections in wash buffer A for 10 min.

Sections were counterstained with light green (1%) (AtomScientific, Manchester, UK) for 4 min, and transferred to a solution of 0.05 % acetic acid in acetone for 1 min. Sections were dipped 10 times in distilled water, dehydrated through increasing concentrations of ethanol (96% and 100%), cleared in xylene and mounted using VectaMount (Vector Laboratories, Burlingame, CA, USA).

2.3 Comparison of the ISH method with other techniques

Atlantic salmon FFPE gill tissue samples were sectioned (4 μm) sequentially and adjacent sections stained with H&E (Stevens and Wilson, 1996) and CW (Fluka, Buchs, Switzerland) (Herrero et al., 2020), as well as using the optimised ISH protocol. All sections were examined with an Olympus BX51 microscope (Olympus, Southend-on-Sea, UK) and photomicrographs were taken with an Olympus DP70 Digital Camera System (Olympus) using analySiS® software (Olympus).

To quantify the number of microsporidia visible in the gill sections, each section was examined using a 20x objective lens over a 10 mm^2 tissue area to determine the number of *D. lepeophtherii* positive structures labelled.

2.4 Assessment of *D. lepeophtherii* presumptive pathology

The characteristic lesions in hypertrophied and necrotic epithelial cells associated with the presence of *D. lepeophtherii* in infected salmon gill tissues have been termed "microvesicles" (Weli *et al*., 2017). The severity of microvesicle/necrosis score (MV/N) on H&E stained sections was scored from 0 to 3: 0 = absence of pathology suggestive of *D. lepeophtherii*, 1 = foci of epithelial cell necrosis (Supplementary Figure 1a) but no obvious *D. lepeophtherii*-related microvesicles, 2 = a small to medium number of microvesicles typical of *D. lepeophtherii* infection, 3 = a large number of microvesicles typical of *D. lepeophtherii* infection present in the gill epithelial cells (Supplementary Figure 1b).

2.5 Statistical analyses

Sensitivity, specificity, positive predictive value and negative predictive value were calculated according to Martin (1977), taking RT-rtPCR results as a reference (Table 3). The data were not normally distributed (Shapiro-Wilk's test) and Spearman rank correlation coefficients were calculated to determine any correlations between the MV/N-H&E score and the Ct values obtained by RT-rtPCR, and between the MV/N-H&E score and the total ISH counts observed in the gill tissue. The Pearson's correlation coefficient test was used to examine correlations between the total ISH counts observed in the gill tissue with the Ct values obtained. Statistical significance was set at $p \leq 0.05$ and all statistical analyses were performed using R (R software, v. 3.5.3; https://www.r- project.org/)

3. RESULTS

3.1 Development and optimisation of the ISH technique

The influence of different concentrations of reagents and variation in incubation times on the success of the ISH protocol are summarised in Table 4. Permeabilization of the tissue with PK for 10 min compared to 30 min gave the same level of labelling of *D. lepeophtherii*, but the tissue morphology was less disrupted after incubation with PK for 10 min, so this was used. Also, omission of the pre-hybridisation step did not affect the results. Cocktail 1 gave slightly more background signal compared to Cocktail 2, although this difference decreased with more stringent washing, i.e. using SSC buffer at 0.25x to remove unbound probes after using Cocktail 1. The optimum dilution of the probes was 1/1000 for both Cocktail 1 and Cocktail 2, with higher concentrations of probes giving no increased signal in the tissue sections. A 3 h incubation time with the AP enzyme substrate was sufficient to produce an optimal labelling, whereas the reaction was over-developed when applied overnight. The final optimised protocol was used to test the probes separately. Probes targeting the SSU rRNA (16L21, 819L25, and 1002L25) produced a strong signal, with some background present using probe 819L25 (Table 2). Probes targeting the ITS region gave different results. Probe 1339L25 gave a good signal whilst probe 1284L21 was the only probe tested that did not show any labelling (Table 2).

3.2 Detection *of D. lepeophtherii* in Atlantic salmon gills using ISH

Successful binding of the probes to the *D. lepeophtherii* target sequences was denoted by a dark blue-purple signal, depicting the presence of the parasite, against a light green counterstain (Figure 1). All negative control preparations were devoid of any blue-purple signal (Figure 1a). Proliferative stages of *D. lepeophtherii*, *i.e.* pre-sporogonic structures and possibly meronts, appeared as intensely blue-purple labelled round structures

297 approximately 4-6 µm in diameter (Figure 1b & 1c) and were present most frequently in
298 the cytoplasm of gill epithelial and blood vessel endothelial cells. Sporont-like structures
299 appeared as a vacuole containing multiple punctate blue-purple inclusions, and were
300 considered to be immature spores (Figures 1d).

301 Both types of spores, auto-infective and environmental, although less consistently
302 labelled than the pre-sporogonic structures were still visibly discernible in the gills. Auto-
303 infective spores were smaller, approximately 0.8-1.0 µm in diameter, and appeared,
304 typically, in clusters in the cytoplasm of gill epithelial cells. These spores did not always
305 appear as complete structures, but were denoted by small punctate labelling in necrotic
306 tissue (Figure 2a). Environmental spores were larger (2.0-2.5µm) and present in the
307 nucleus of gill epithelial cells or associated with necrotic epithelial cells (Figure 2b).
308 Spores were generally less intensely labelled than pre-sporogonic stages and variation in
309 the intensity was noted also (Figure 2c).

310 **3.3 Detection of *D. lepeophtherii* in non-gill tissues and probe specificity**

311 Tissues from the RT-rtPCR-positive fish also showed labelling for *D. lepeophtherii* by ISH
312 in the kidney interstitium (Figure 3a), the parenchyma of the spleen and liver and *lamina*
313 *propria* of the intestine (Figure 3b). Sections of sea lice containing typical xenoma-like
314 structures, caused by *D. lepeophtherii*, were also ISH positive, with multiple foci of
315 microsporidia labelled (Figure 3c). The gills of Atlantic salmon from the Canadian fish
316 farm showed similar labelling to those from Scottish farms using the probes targeting the
317 SSU rRNA (Figure 3d), but no labelling was obtained with probes complementary to the
318 ITS region. The oligoprobes used in this study did not cross-react with any of closely related
319 microsporidian species examined (*N. cyclopterii* and *E. hepatopenaei*) as denoted by the
320 tissue sections containing them being devoid of any labelling after being subjected to the
321 ISH protocol.

3.4 Comparison of ISH with other techniques used to identify *D. lepeophtherii*

Calcofluor white stained two different sizes of spores both of oval/spherical shape. The smaller one (1.1 μm length) was present in the cytoplasm of cells along the lamellae, but the specific cell types were difficult to identify due to the poor preservation of tissue morphology associated with this technique. These smaller spores were mostly present in aggregates of 3 to 20; when not present in aggregates a 60x objective lens was required to visualise individual spores due to their low level of fluorescence (Figure 4).

Larger, oval, spores were clearly visible under a 20x objective due to their stronger fluorescence signal and larger size (~2.5 μm-length). These were present singularly or in pairs. It was unclear if they were located within the nucleus or the cytoplasm of the cells, again, due to the poor preservation of tissue morphology that occurs with this technique. Calcofluor white stained large (environmental) spores in gill tissue even when parasite loads were low (as denoted by RT-rtPCR), but the smaller auto-infective spores were only visible with CW when the total number of microsporidia labelled with ISH was high ($\geq$150/10 mm^2).

Results for the 28 different sections of Atlantic salmon gills analysed using ISH, CW and H&E (based on the presence of microvesicles) showed that all methods were highly specific (100%) in their ability to detect *D. lepeophtherii* but that the sensitivity, compared to RT-rtPCR, was markedly higher using the ISH technique (92%), followed by CW (64%) and then H&E (52%) (Table 5). Positive predictive values (probability that subjects with a positive screening test really have the disease) and negative predictive value (probability that subjects with a negative screening test do not have the disease) are also shown in Table 5.

3.5 Gill *D. lepeophtherii* burden and the presence of microvesicles

From the 25 *D. lepeophtherii* RT-rtPCR-positive fish examined, 6 were devoid of any pathology suggestive of *D. lepeophtherii* infection (MV/N-H&E score 0). Presence of mild to moderate, multifocal, lamellar epithelial cell necrosis (Figure 5) was present in 5 fish (MV/N-H&E score-1). Low numbers of microvesicles, suggestive of *D. lepeophtherii*, were present in 11 of the examined gill samples (MV/N-H&E score 2), and a large number microvesicles were present in 3 fish (MV/N-H&E score 3). The presence of microvesicles was mostly observed when the total number of *D. lepeophtherii* labelled by ISH was between 120-850/10 mm^2 tissue in fish gill tissue, although one fish had a small number of microvesicles present despite having only 30 ISH-positive *D. lepeophtherii* structures (Figure 6). In agreement with this, the presence of microvesicles in gill tissue sections was observed only when the *D. lepeophtherii* load was very high as determined by RT-rtPCR (Ct ≤19). Swabs taken from gills and assessed by RT-rtPCR showed less consistent results with respect to the presence of necrosis in the corresponding tissue sections when examined histologically. The swab from fish 23 had a Ct value of 19 but was devoid of necrosis in the corresponding gill tissue section stained with H&E, while the gill swab from fish 24 had a Ct value of 20 but had large numbers of microvesicles in its corresponding tissue section, visible by H&E staining, suggestive of *D. lepeophtherii* infection.

Spearman rank correlation coefficient between the MV/N - H&E score and the total ISH counts in the gill tissue was significant (rs= 0.89; 95% confidence interval; $p < 0.001$). There was a significant correlation between the MV/N-H&E score and Ct values obtained from gill biopsies (rs= -0.92; 95% confidence interval; $p < 0.001$), but there was no correlation between the MV/N-H&E score and the Ct results obtained from gill swabs (rs= 0.69; 95% confidence interval; p= 0.056). When the total number of microsporidia labelled with ISH was compared with all RT-rtPCR results (from gill biopsies and swabs) there was a significant correlation ($p \leq 0.03$), and between the ISH and the gill biopsy Ct value results

372 ($p \leq 0.03$). However, there was no correlation ($p = 0.22$) between the ISH scores and the Ct
373 results from gill swabs.
374
375 **4. DISCUSSION**
376 A total of five species-specific antisense DNA oligonucleotide probes were designed for *D.*
377 *lepeophtherii,* three complementary to the positive strand of the SSU and two to the ITS
378 region. Two of the three probes directed against the SSU and one to the ITS region gave a
379 strong positive signal in the ISH, while labelling with the other SSU probe, 819L25, did
380 not give clear visualisation of the parasite due to non-specific labelling. Reduction of the
381 non-specific labelling when using probe 819L25 was achieved by increasing the stringency
382 of washing steps (Wilcox, 1993) thereby removing effectively the all unbound probe. As
383 well as detecting *D. lepeophtherii* in the gills of farmed salmon from Europe, the four
384 functional probes also detected *D. lepeophtherii* in the gills of Canadian farmed salmon.
385 The only probe that did not label *D. lepeophtherii* was 1284L21, which had several
386 mismatches with the Canadian genotype as it was designed deliberately to discriminate (in
387 the ITS region) between the European and Canadian genotypes of *D. lepeophtherii*.
388 However, this design was based on only two available sequences representing both
389 genotypes and extending across the ITS and greater diversity may exist in *D. lepeophtherii*
390 genotypes at this locus. In the fungal kingdom, the ITS region has been shown to be
391 generally better for inter- and intraspecific discrimination compared to the LSU or SSU
392 (Schoch et al., 2012). For instance, more than 100 genotypes have been described for the
393 microsporidian *Enterocytozoon bieneusi*, a microsporidian closely related to *D.*
394 *lepeophtherii*, by sequence analysis of the ITS, and these various genotypes have been
395 associated with different host affinities and different levels of pathogenicity (Galván-Díaz
396 et al., 2014). Further molecular characterization of intraspecies genetic diversity between

D. lepeophtherii obtained from different geographical areas and host species may help in understanding the role of this microsporidian species in gill disease.

The probes we developed for the ISH appear to be specific for *Desmozoon* spp. in that they did not cross-react with the two related microsporidia examined (*E. hepatopenaei* and *N. cyclopterii*). Also, the xenoma-like structures found below the cuticle of sea lice, highly suggestive of a *D. lepeophtherii* infection (Freeman and Sommerville, 2009), labelled intensely when screened with the ISH protocol confirming the presence of the parasite. Therefore, the ISH will be a valuable tool for studying the various stages in the life cycle of *D. lepeophtherii* both in sea lice and Atlantic salmon.

In the gill tissue, positive labelling, by ISH, of the parasite's developmental stages was present in the cytoplasm and nuclei of the gill lamellar epithelial cells and in the cytoplasm of endothelial cells of the blood vessels in the gills in agreement with the described life cycle (Nylund et al., 2010) and previous studies (Weli et al., 2017). Small auto-infective spores were sometimes present within the cytoplasm of apparently normal gill lamellar epithelial cells, in the absence of pathology, while larger environmental spores were occasionally observed within nuclei of gill lamellar epithelial cells, or associated with degenerate epithelial cells, and both spore types had variable and less intense labelling compared to the pre-sporogonic stages. Limitations of ISH in detecting spores are reported frequently and considered to be due the predicted lower number of targets and the low permeability of microsporidian spore stages to probes (Ahmed et al., 2019). Additionally, the detection of microsporidia with ISH using antisense DNA oligonucleotides that target the SSU region may result in a poor spore signal due to reduced or absent target protein synthesis during the spore stage and the highly condensed genome of some microsporidian species which could reduce the availability to the probes of the target regions in the parasite's genomic rDNA. This reduced intensity of labelling of

spores by ISH has been reported for other microsporidian species including a RNA-based ISH method for detecting *D. lepeophtherii* (Weli et al., 2017). In our procedure, spores were identified mainly as punctate labelling and rarely as fully labelled oval structures. However, the increased intensity of ISH spore signal in this study compared to other studies could be due to the use of the 5′-, 3′-doubly labelled probes employed instead of the more typical singly labelled probes. This approach has proven successful in other studies (Stoecker et al., 2010).

Although a description of the parasite's systemic distribution and associated pathology was not the aim of this study, fish with high burdens of *D. lepepophtherii* in the gills (by ISH signal or RT-rtPCR values) showed a positive ISH signal in other organs. *Desmozoon lepeophtherii* was first detected in salmon tissue by conventional PCR in the kidney, liver, gills and circulating blood cells from (clinically normal) farmed Atlantic salmon in Scotland (Freeman, 2002). Later, Nylund et al. (2010) detected developmental stages of the parasite in the blood vessel endothelial cells and the cytoplasm of leukocytes by transmission electron microscopy. The systemic distribution of the microsporidian has been reported by others (Matthews et al., 2013; Nylund et al., 2011) but has not been associated with major tissue damage with the exception of one case report in which the presence of *D. lepeophtherii* was associated with severe pathology in the gills, peritoneal cavity and the gastrointestinal tract (Weli et al., 2017). In our study, only low levels of *D. lepeophtherii* were detected in the gastrointestinal epithelium. However, we only used single or small groups of fish from different clinical cases, which may not have had the same clinical signs as those reported by Weli et al. (2017). Little is known about the effects of *D. lepeophtherii* in the other organs of fish and its systemic distribution has generally been overlooked.

Analysis of the sensitivity and specificity of the ISH protocol allowed comparison with other detection methods. Complete clinical validation of this ISH method would require the analysis of samples from a large population of *D. lepeophtherii*-infected *vs.* non-infected fish (Georgiadis et al., 1998). Unfortunately, sampling of the required magnitude was outwith the scope of the present study. In addition, the peak incidence of clinical disease is usually seasonal and short (Matthews et al., 2013), and a large number of tissue samples from fish with typical gross pathology would be challenging to collect. However, gill samples from 28 fish subjected to the ISH protocol allowed initial comparison with other histological methods (CW and H&E) commonly used to detect *D. lepeophtherii*. Tissue sections containing various parasite loads were selected based on RT-rtPCR results, used as a 'gold standard positive control'. The sensitivity of the ISH protocol was high (92%) when compared to RT-rtPCR. As both techniques target the genome of the microsporidian, both the PCR and the ISH are capable of detecting levels associated with subclinical as well as clinical infections that can be missed by other, less sensitive, histological methods. Although RT-rtPCR is more sensitive than ISH, because of the amplification of the original signal, it is susceptible to false positive results due to contamination. The ISH protocol combines the high sensitivity and specificity of molecular detection with direct observation of the presence, subjective load and distribution of the parasite, including the severity and morphology of histological lesions in the gill tissue and parasite lesion-association. Furthermore, when gill biopsies where subjected to RT-rtPCR, the Ct values obtained correlated significantly with the total number of parasites observed in the gill tissue using ISH. However, no correlation was found between RT-rtPCR Ct values derived from gill swabs and the ISH of the corresponding gill tissue. These results highlight the usefulness of ISH for quantifying the parasite level and for determining the parasite-associated pathology in the gills, which

facilitates the understanding of the role of *D. lepeophtherii* in gill disease. Additionally, due to its high specificity and sensitivity the ISH method can be used as a standalone procedure in the absence of RT-rtPCR results. Although gill swabbing is a minimally invasive option for assessing the presence of the microsporidian in fish gills by RT-rtPCR, our results show using gill swabs is less reliable than using gill tissue, probably because of the intracellular nature of the parasite.

Calcofluor white allowed the visualization of the two types of spores described for *D. lepeophtherii* (Nylund et al., 2010), in agreement with previous studies (Herrero et al., 2020; Weli et al., 2017), and has been widely used to detect microsporidia (Luna et al., 1995). This is due to its ability to bind to chitin present on the inner layer of the spore wall and its greater sensitivity compared with other routine histological techniques (Herrero et al., 2020). However, the sensitivity of CW was only 64% when compared to RT-rtPCR, probably because the pre-sporogonic stages, which develop prior to the spores, are not detected by CW. Assessment of *D. lepeophtherii* based on the presence of microvesicles in gill tissue sections stained with H&E gave the lowest sensitivity (52%). Microvesicles were only detected in 14 of the 28 fish examined, while the absence of *D. lepeophtherii*-related necrosis was recorded in 8 fish. High burdens of *D. lepeophtherii* in tissue sections, as denoted by Ct values below 19 and/or ISH total counts of over 100 microsporidia/10 mm^2 of gill tissue, were significantly associated with the development of the microvesicles and these high burdens seem to be necessary for their formation. Microvesicles caused by the microsporidian are probably the consequence of intense parasite proliferation and spore formation and only obvious in the advanced and/or severe stages of the disease, likely due to the release of the spores and disruption of fish cells. The presence of necrosis of epithelial cells but absence of microvesicles, denoted in this study as foci of epithelial cell necrosis, was suspected to be an early stage of the microvesicle formation.

Nevertheless, this change is non-specific and was not consistently associated with the presence of a positive ISH signal. Although the presence of medium to high numbers of microvesicles are highly suggestive of *D. lepeophtherii* infections in Atlantic salmon gills, a positive RT-rtPCR or ISH result is necessary to confirm the presence of the parasite in clinical cases.

The DNA-based ISH protocol developed during this study effectively detects *D. lepeophtherii*, especially the pre-sporogonic phase, which does not label with other histological techniques in Atlantic salmon in FFPE tissue sections. Therefore, this technique enables assessment of parasite burden such that it correlates significantly with the RT-rtPCR results in the absence of microvesicles which are present typically when the *D. lepeophtherii* burden in gill tissue was very high, as determined by RT-rtPCR (Ct values $\leq$ 19). Gill diseases are often complex and other pathology associated with the microsporidian, such as epithelial cell necrosis, epithelial cell proliferation and inflammation in the gills, is non-specific and can be associated with other infectious or environmental factors. In addition, *D. lepeophtherii*-associated pathology changes during disease progression (Matthews et al., 2013; Weli et al., 2017). Therefore, unless severe the pathology caused specifically by *D. lepeophtherii* is difficult to discern in a complex gill disease scenario by conventional H&E.

In conclusion, the ability of this newly developed ISH method to detect lesion-associated *D. lepeophtherii* with very high sensitivity and specificity will provide valuable information on the infection dynamics of the parasite. This will make it critical for studying the progressive development and spread of *D. lepeophtherii* and possibly help establish a suitable challenge model by either feeding fish infected tissue or cohabiting infected fish with naïve fish. As this ISH protocol detects both spore types and the pre-sporogonic phase of the parasite's infection in salmon gills and other tissues, it will enable a better

521 understanding of the parasite's life cycle and identification of the specific associated
522 pathology.
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TABLE 1. Sequences of *D. lepeophtherii* recovered from different host species and countries of origin, sequences were aligned to exclude variable regions in the oligonucleotide probe design. 16S small subunit (SSU) and internal transcribed spacer (ITS), 23S large subunit (LSU).

Accession Number	Number of bp	Target Gene	Host species/ Country
FJ594990	1885	SSU (partial)	Female sea lice *L. salmonis* / Norway
FJ389667	1656	SSU and LSU (partial); ITS;(complete)	Farmed Atlantic salmon / Norway
HM800847.2	1826	SSU (partial)	Farmed Atlantic salmon /Canada
AJ431366.2	1787	SSU; ITS; LSU (partial)	*L. salmonis* /Scotland
KR187183	1584	SSU (partial)	Wild rock cook wrasse (*Centrolabrus exoletus*)/ Norway
FJ594979	1559	SSU (partial)	*Caligus elongatus*/ Norway
FJ594989	953	SSU (partial)	Farmed rainbow trout/ Norway
HM367691	685	SSU (partial)	Wild Atlantic salmon/ Norway

TABLE 2. Oligoprobe sequences specific for *Desmozoon lepeophtherii* designed for the *in situ* hybridization protocol. SSU = small subunit ribosomal ribonucleic acid, ITS = internal transcribed spacer, Tm = melting temperature of the probes. Results for individual oligoprobes in the optimised ISH protocol: - absence of labelling, + strong labelling, BL background labelling.

Name	Sequence 5'-3'	Region	Tm	Results
1284L21	CAAATCTGAACGTGATGCTAT	ITS	62.5°C	-
16L21	CGTTCCCCATTCGGTTCACAG	SSU	69.8°C	+
819L25	TTGCCCCTCTCATGTCGCCAATCTA	SSU	74.4°C	+, BL
1002L25	ATATTTATGTCGCTCAAACGGATA	SSU	64.5°C	+
1339L25	ACACACTCACTAAGCAGTCCTACTA	ITS	69.1°C	+

TABLE 3. Formulae used to calculate the sensitivity and specificity of the various *Desmozoon lepeophtherii* detection techniques, using RT-rtPCR as reference.

Calculation	Formula
Sensitivity	∑true positive results/∑true positive samples *100
Specificity	∑true negative results/∑true negative samples*100
Positive predictive value	∑true positive results /∑ true and false positive results*100
Negative predictive value	∑true positive results /∑ true and false negative results*100

TABLE 4. Influence of different concentrations of reagents and variation in incubation times during optimisation of the ISH protocol. +/- weak signal, + strong signal, BL background labelling, SBL strong background labelling. Cocktail 1 (C1) consisted of probes 16L21, 819L25 & 1339L25. Cocktail 2 (C2) consisted of probes 1284L21, 1002L25 & 1339L25.

Step	Duration	Results
Proteinase K	15 µg mL^{-1} 10 min	C1: +, BL
		C2: +
	15 µg mL^{-1} 30 min	C1: +, BL
		C2: +
	No proteinase K	C1: +/-, BL
		C2: +/-
Pre-hybridization	30 min	C1: +, BL
		C2: +
	None	C1: +, BL
		C2: +
Stringency washes	2x SSC, 10 min 1x SSC, 10 min 0.5x SSC	C1: +, SBL
		C2: +
	10 min 2x SSC, 10 min 1x SSC, 10 min 0.25x SSC	C1: +, BL
		C2: +
Substrate incubation	3 h	C1: +, BL
		C2: +
	Overnight	C1: SBL
		C2: SBL

TABLE 5. Sensitivity, Specificity, Positive predictive value (PPV) and Negative predictive value (NPV) of the techniques used when compared with the RT- qPCR results for detecting the presence of *Desmozoon lepeophtherii* in salmon gills.

Method	Analysis	Results	Analysis	Results
In situ hybridization	Sensitivity	92.0%	PPV	100.0%
	Specificity	100.0%	NPV	60.0%
Calcofluor White	Sensitivity	64.0%	PPV	100.0%
	Specificity	100.0%	NPV	25.0%
Microvesicles (H&E)	Sensitivity	56.0%	PPV	100.0%
	Specificity	100.0%	NPV	21.4%

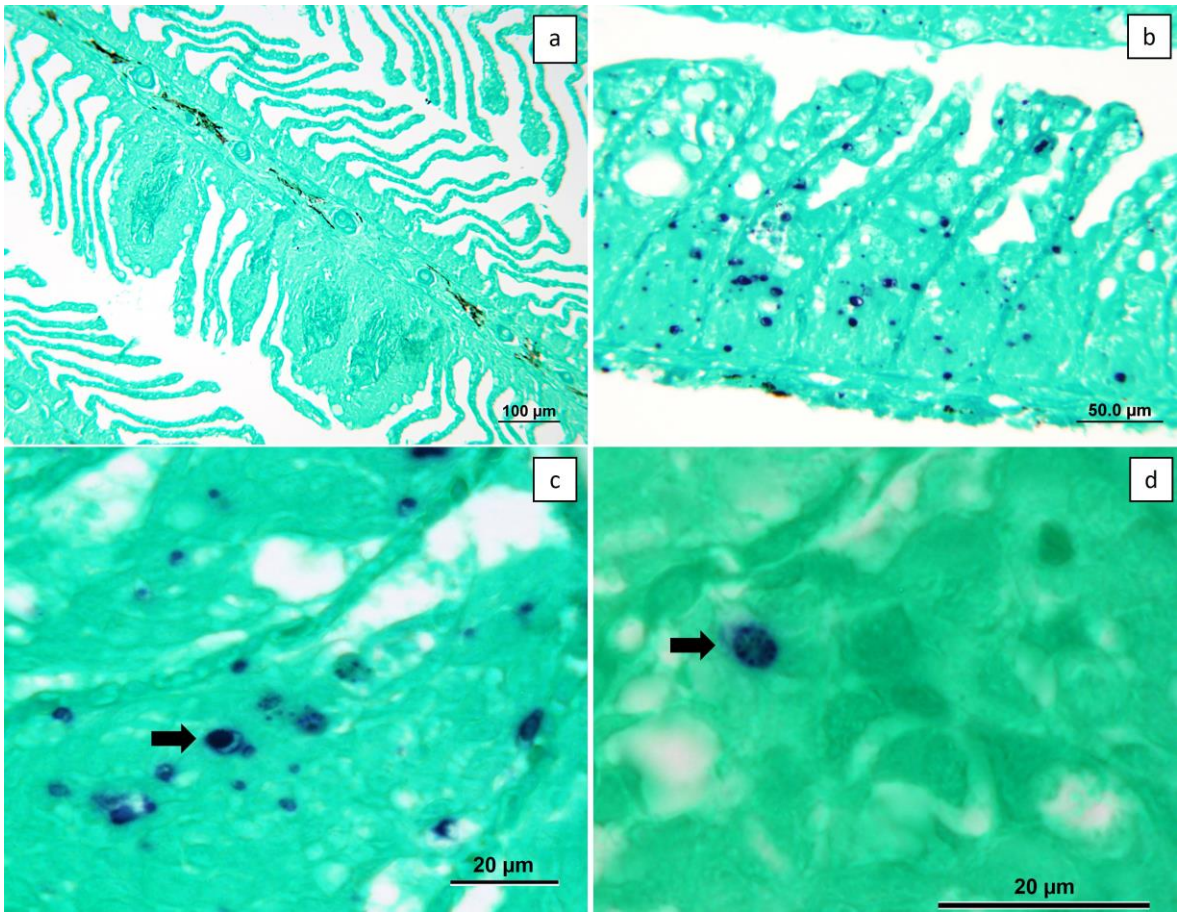

FIGURE 1. Atlantic salmon gill tissues subjected to *in situ* hybridisation specific for *Desmozoon lepeophtherii* (dark blue/purple pigment). (a) Negative for *D. lepeophtherii*, note section is devoid of labelling. There is melanin (dark brown pigment) in filament central venous sinus. (b) Positive, note pre-sporogonic stages present in the epithelial cells of the gill lamellae. Dark brown pigment in filament indicates melanin. (c) Positive, note the meront-like structure present (arrow) approximately 4 µm in diameter. (d) Positive, note sporont-like structure with punctate labelling that corresponds to forming spores.

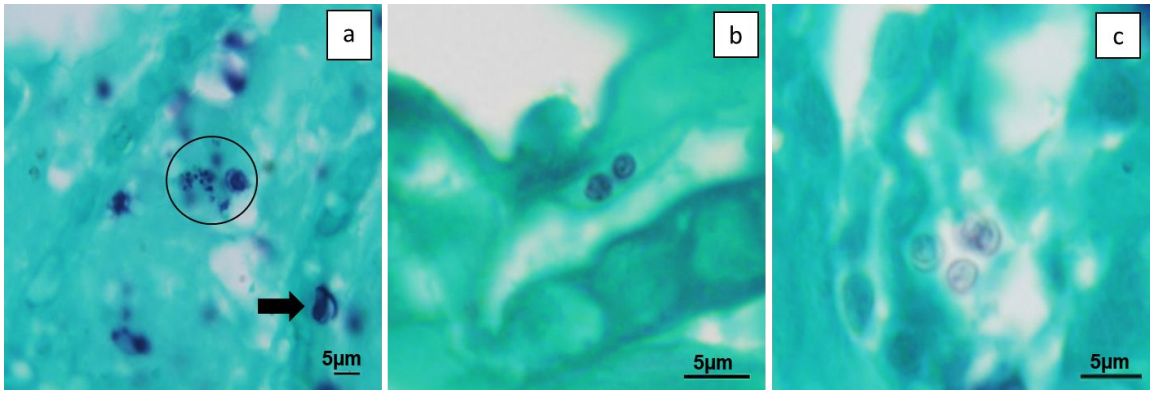

FIGURE 2. *In situ* hybridisation showing the presence of *Desmozoon lepeophtherii* in the gills of Atlantic salmon (dark blue/purple pigment). (a) Note proliferative stages (arrow) and a cluster of spore-like structures (circle) within the proliferating epithelium of the gill lamella; (b) Two labelled environmental spores; (c) A group of poorly labelled environmental spores of *D. lepeophtherii* measuring 2.5µm in diameter.

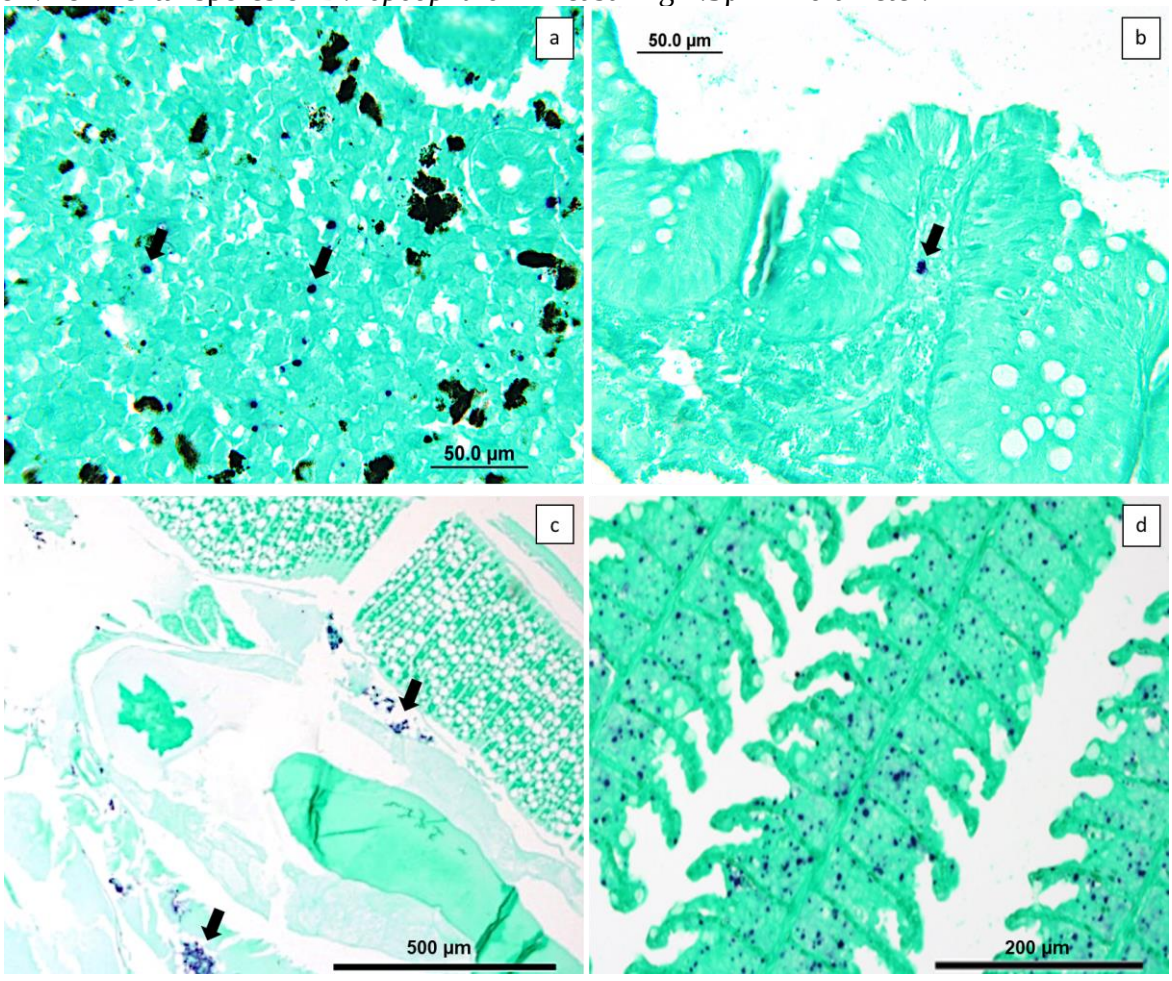

FIGURE 3. *In situ* hybridisation for *Desmozoon lepeophtherii* (dark blue/purple pigment) showing proliferative stages in (a) kidney interstitium (arrows) and (b) the *lamina propria* (arrow) of the intestine of Atlantic salmon; (c) sea louse (*Lepeophtheirus salmonis*) infected with *Desmozoon lepeophtherii* (arrows) and (d) gills from an Atlantic salmon from a salmon farm in Canada heavily infected with *D. lepeophtherii* (arrow). There is melanin (dark brown pigment) in kidney interstitium.

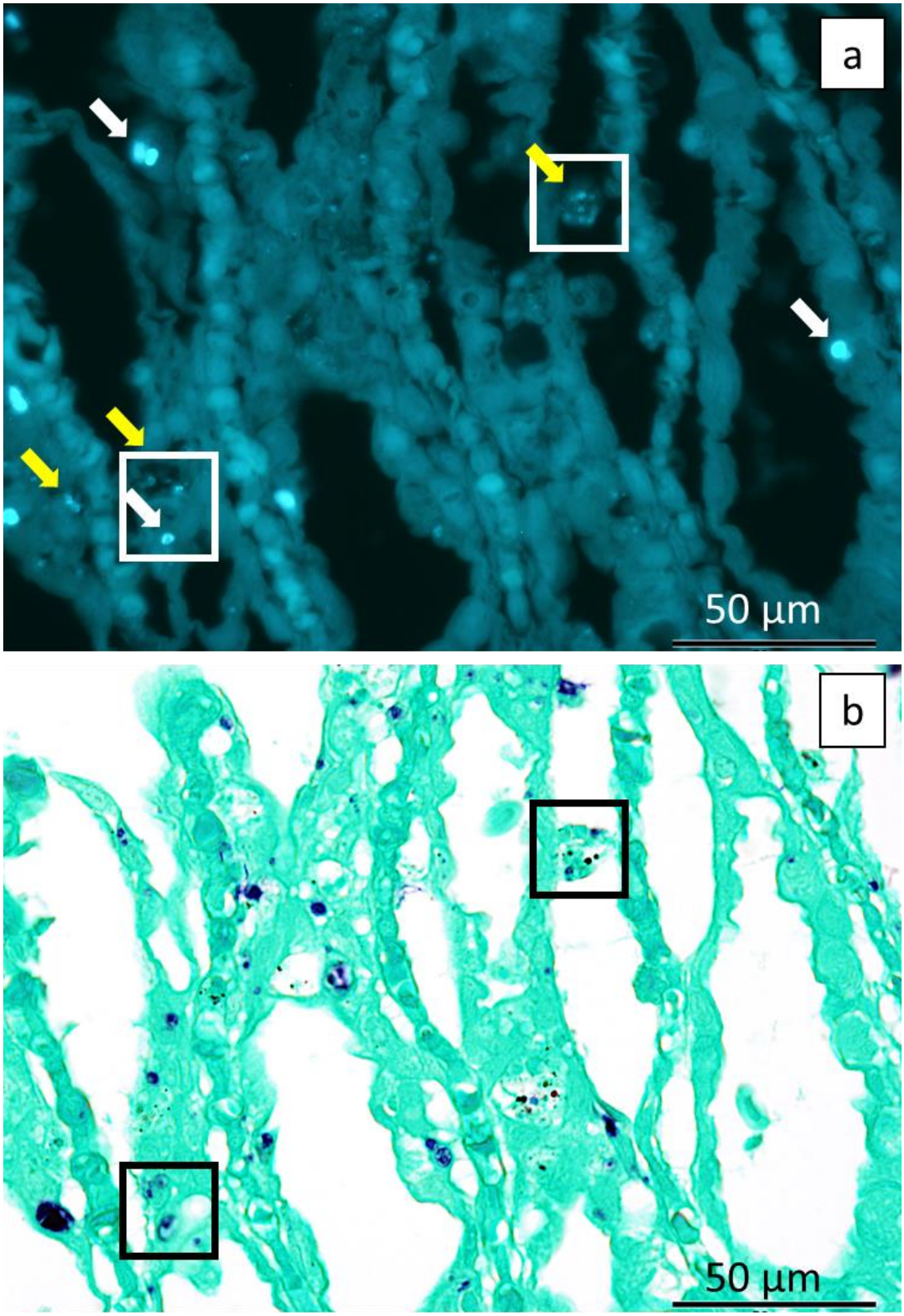

FIGURE 4. Semi-serial histological sections of gills of *Salmo salar* infected with *Desmozoon lepeophtherii*. (a) CW showing bright structures corresponding to large (white arrows) and small (yellow arrows) microsporidian spores, (b) note the same structures label (dark blue/purple pigment) with ISH (boxes).

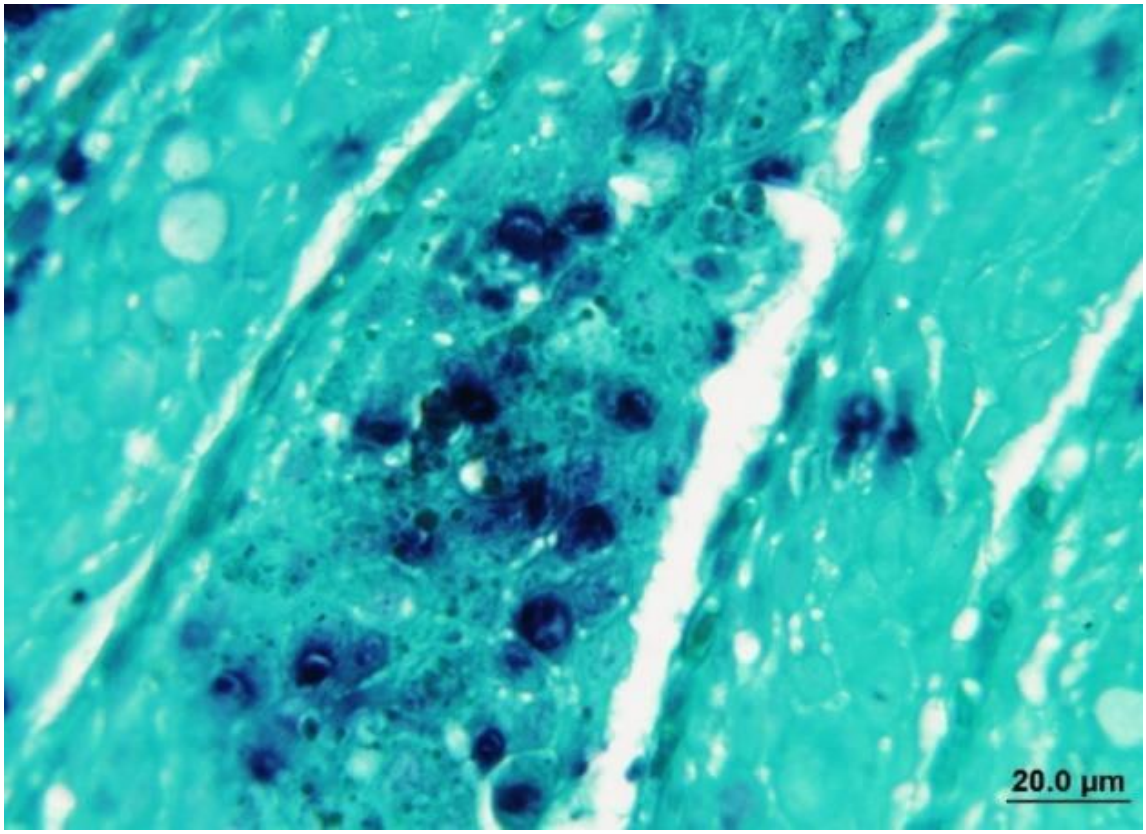

FIGURE 5. *In situ* hybridisation showing the presence of *Desmozoon lepeophtherii* (dark blue/purple pigment) in the gills of *Salmo salar* associated with a foci of epithelial cell necrosis.

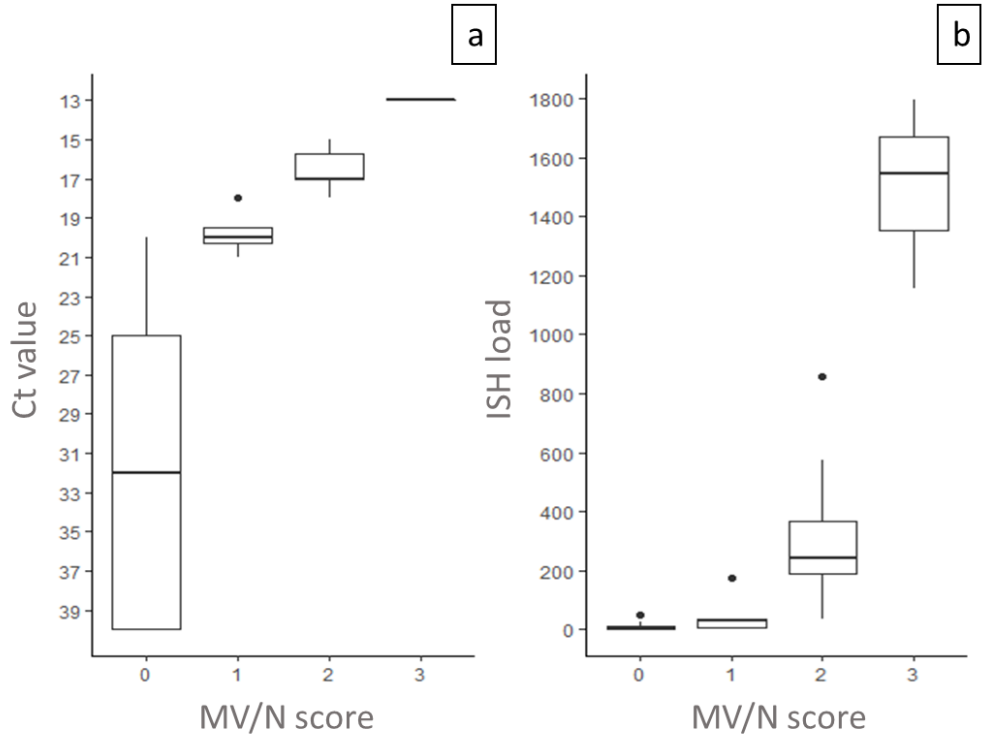

FIGURE 6. Boxplot of the microvesicle/necrosis score (MV/N) in salmon gill tissue with different burdens of *Desmozoon lepeophtherii* represented as (a) RT-rtPCR Ct values and (b) ISH total counts in 10 mm² of gill tissue (ISH load). Microvesicle/necrosis score (x-axis): 0 absence of necrosis, 1 epithelial cell necrosis but absence of microvesicles, 2 presence of small to medium numbers of microvesicles, 3 large numbers of microvesicles.