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Co-infection of *Nucleospora cyclopteri* (Microsporidia) and *Kudoa islandica* (Myxozoa) in farmed lumpfish, *Cyclopterus lumpus* L., in Norway: a case report

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Abstract

This study describes a co-infection of *Kudoa islandica* (Myxozoa) and *Nucleospora cyclopteri* (Microsporida) in farmed lumpfish, *Cyclopterus lumpus* L., in Norway. Several other parasites (*Cryptocotyle* sp., protozoan ciliates and *Gyrodactylus* sp.) were also found in gills. In June 2013, the mortality in a farmed lumpfish population increased to 65%. Lumpfish showed erratic swimming behaviour and loss of weight. At necropsy, nodules in the kidney were the only visible lesions. Histologically, all fish showed severe changes with gill inflammation and necrosis in the spleen, kidney and liver. Haemorrhages and necrosis were observed in some hearts. Intracellular microsporidians associated with the lesions were detected in most organs using histological examination and Calcofluor White. *Kudoa* spores were diagnosed in the skeletal muscle, but no inflammatory response was associated with the presence of the plasmodia. Comparison of 18S ribosomal DNA sequences showed 100% similarity to *Kudoa islandica* and *Nucleospora cyclopteri*. *Kudoa islandica* and *N. cyclopteri* have previously been described associated with lesions in wild lumpfish in Iceland. In the present case, *N. cyclopteri* is believed to be the main cause of systemic pathology. This is the first description of *K. islandica* and *N. cyclopteri* causing pathology in farmed lumpfish in Norway.

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Introduction

Lumpfish or lumpsucker, *Cyclopterus lumpus* L. (Order: Scorpaeniformes), is widely distributed in the North Atlantic and is an important commercial species in Iceland, Greenland, Canada, Denmark, Sweden and Norway. In recent years, the use of lumpfish as cleaner fish or biological agent for salmon lice (*Lepeophtheirus salmonis* Krøyer) control has increased considerably in fish farms in Norway. The salmon farming industry has a huge challenge in controlling the number of salmon lice, and as salmon lice develop resistance to chemical compounds, other non-chemical strategies are being implemented (Torrissen *et al.* 2013; Imsland *et al.* 2014). Traditionally, the cleaner fish used in salmon farms were wild-caught, but due to increased demand, commercial farms have been established. More than fifteen million cleaner fish (lumpfish and wrasse) were used in Norwegian salmon farms in 2013; approximately two million of these fish were farmed (Anonymous 2014). One of the biggest challenges in the farming of new species is the control of diseases. Many parasites and bacterial infections have been diagnosed in lumpfish (Poppe *et al.* 2012, 2013; Johansen 2013; Marcos-López *et al.* 2013; Hjeltnes 2014; Karlsbakk *et al.* 2014). So far, no viral diseases have been confirmed, but it has been experimentally shown the possibility of infectious pancreas necrosis (IPN) virus to infect lumpfish

Journal of Fish Diseases

(Wesmajervi Breiland and Johansen, personal communication).

Myxosporeans in the genus *Kudoa* have been described from many different marine and estuarine fishes worldwide (Moran, Whitaker & Kent 1999; Eiras, Saraiva & Cruz 2014). Most species are found intracellularly in muscle cells, but can also be observed in other organs (Lom & Dykova 2006). The infections are often causing 'soft flesh' or post-mortem myoliquefaction, rendering the fish unsuitable for consumption. Several species of *Kudoa* infect single fish species, but species such as *K. thyrsites* and *K. islandica* have been shown to be less host specific. *Kudoa thyrsites* (Gilchrist, 1924) infects as many as 38 fish species worldwide including farmed Atlantic salmon (*Salmo salar* L.) in North America. In Norway, *K. thyrsites* is reported from Atlantic mackerel (*Scomber scombrus* L.) as the only *Kudoa* species to date (Levsen, Jørgensen & Mo 2008). Recently, a new species, *K. islandica,* was described from Iceland infecting the muscle of, Atlantic wolffish (*Anarhichas lupus*), spotted wolffish (*A. minor*) and wild lumpfish, the latter being the most heavily parasitized species (Kristmundsson & Freeman 2014).

Microsporidians are obligate intracellular parasites (Lee *et al.* 2008) which mostly infect fish and arthropods although they have been described parasitizing almost all animal groups, from invertebrates to humans. *Nucleospora cyclopteri* (Family: Enterocytozoonidae) was recently described from wild lumpfish in Iceland (Freeman, Kasper & Kristmundsson 2013) and has also been reported from wild lumpfish in Norway (Karlsbakk *et al.* 2014). The same species was also suspected to be the cause of chronic mortalities in cultured lumpfish in Canada (Mullins *et al.* 1994; Freeman *et al.* 2013). In both Icelandic and Canadian cases, the parasites were mainly found as intranuclear parasites in lymphocytes and lymphocytes precursor cells. The same cells are also affected in infections with *N. salmonis* (Georgiadis, Gardner & Hedrick 1998), a closely related parasite affecting salmonids.

Desmozoon lepeophtherii, a microsporidian infecting both salmon louse and Atlantic salmon in Scotland, Norway and North America (Freeman, Bell & Sommerville 2003; Freeman & Sommerville 2009), is often detected in cases of chronic gill inflammation together with other pathogens (Steinum *et al.* 2010; Nylund *et al.* 2011). In previous studies, *D. lepeophtherii* was present in clinically healthy salmon, but the load of microsporidians was substantially higher in fish with gill lesions. It is not known whether *D. lepeophtherii* can suppress host immune response or whether it is secondary to other infections in already suppressed fish, as has previously been suggested for other microsporidia (Laudan, Stolen & Cali 1986; Wongtavatchaia, Conrad & Hedrick 1995; Freeman & Sommerville 2011).

In the present study, we describe for the first time a *K. islandica* infection in farmed lumpfish in Norway. In addition, we describe gill and systemic pathology caused by the microsporidian *N. cyclopteri* and comment on other gill parasites (*Gyrodactylus* sp., *Cryptocotyle* sp., protozoan ciliates) infecting the same fish. Similarities and differences with previous reports of *K. islandica* and *N. cyclopteri* in wild lumpfish are discussed.

Materials and methods

Fish population studied

Sexually mature wild lumpfish were caught locally in northern Norway with nets during early spring 2011. The fish were kept in 5-m tanks with water temperature of 12.5 °C. Natural spawning occurred at the end of May, and first generation of farmed juveniles hatched at around 240 degree days. The juveniles were initially fed by hand with natural plankton while adapting to dry feed pellets. The lumpfish juveniles (average weight 7, 8 g) were transferred to a net pen (5 m^3) research facility in sea in November 2011. During 2012, the lumpfish were used to assess delousing efficacy and were also used as part of further studies in the summer 2013 when mortalities occurred. At 2013 trial start (week 16), Atlantic salmon (500 ± 15% i.e. 425–575 g) were bulk weighed, counted and distributed between two cages of 125 m3 (5 m^3) with 120 fish in each cage (n = 120; N = 240). Eighteen lumpfish (mean weight 450 g) were stocked into each of the cages containing salmon, thus establishing duplicate treatments for each population. Each cage containing lumpfish had a ratio of salmon to lumpfish of 7:1. In late June, mortality rates of the lumpfish increased and samples were taken to establish the cause of death. After sampling, the remaining lumpfish in the cage were killed.

Histology

Ten adult lumpfish (500 g) were sampled by the local fish health services and submitted to the Norwegian Veterinary Institute for diagnostic investigation. Tissue samples from gills, skeletal muscle, heart, pyloric caeca, pancreas, kidney, spleen and liver were fixed in 10% neutral phosphate-buffered formalin. After at least 24-h fixation, the samples were processed and embedded in paraffin wax according to standard procedures. Sections were stained with haematoxylin and eosin (H&E) and examined by light microscopy. Selected sections were also stained with Periodic acid–Schiff (PAS) for carbohydrates, Giemsa and May Grünwald Giemsa for parasites, Gram for bacteria and von Kossa for calcium (Bancroft & Stevens 1990). Selected slides were deparaffinized and stained with the fluorochrome Calcofluor White M2R (Sigma-Aldrich), a stain specifically binding to cellulose and chitin. Images were generated on a Zeiss LSM 710 confocal scanning laser microscope, using a 405-nm laser to excite the fluorochrome.

Molecular analyses

PCR and DNA sequencing were applied to the samples for identification and not to establish the prevalence of infection. Thus, not all positive samples were necessarily subjected to sequencing. DNA was extracted from gills and hearts of all ten fish using the DNeasy® Tissue Kit protocol for animal tissues or the QiaQube robot (Qiagen). All PCRs were performed using illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare) with an annealing temperature of 55 °C. PCR targeting the small subunit (SSU) region of the ribosomal array of *Nuclespora cyclopteri* was carried out using the primer pairs LN1_fwd/LN1_rev and LN2_fwd/LN2_rev (Freeman *et al.* 2013) amplifying 950 bp and 590 bp, respectively. The second primer pair was used in a nested PCR using 1 μL of PCR product from the first reaction as a template. PCR targeting amplification of *c.* 650 bp of the small subunit (SSU) region of the ribosomal array of *Kudoa islandica* was carried out using the primer pair Kud-80f/Kud-730rev (Kristmundsson *et al.*, 2014). PCR targeting the ribosomal internal transcribed spacer 2 (ITS2) (*c.* 450 bp) of *Gyrodactylus* spp. was carried out on gill samples using the primer pair ITS2/ITS4.5 (Matejusová *et al.* 2001).

DNA sequencing

The PCR products were cleaned with ExoSAP-IT® (Affymetrix Inc.) and then sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit. As the PCR using the Kud-80f/Kud-730rev primer pair amplified more than one product, bands corresponding to correct fragment sizes were excised from the gel and cleaned using Nucleo-Spin Gel and PCR Clean-up Macherey–Nagel gel extraction kit. The sequencing (both sense and antisense strands) was carried out using the amplification primers. The sequence data were assembled with the Vector NTI 11 software (Invitrogen) and subjected to BLASTN searches in GenBank for similar sequences.

In addition to the parasite-specific PCRs described above, all samples were tested for the following viruses: piscine nodavirus, salmonid alphavirus (SAV), infectious pancreatic necrosis virus (IPNV) and viral haemorrhagic septicaemia virus (VHSV) during routine diagnostics using established real-time PCR assays.

Results

Clinical signs and macroscopic changes

Mortality increased 18 months after sea transfer, and some lumpfish showed erratic swimming behaviour near the surface and evident loss of weight. The mortality of the lumpfish after June 2013 increased to 65%. At necropsy, white nodules in the kidney in two of ten fish were the only described lesions. The salmon stocked in the same cages showed no mortality or abnormal behaviour and feeding response and growth was normal.

Histology

Skeletal muscle. The most common lesion was a moderate infection with *Kudoa* in skeletal muscle in five of ten analysed fish. In the affected muscle fibres, the sarcolemma was partially or completely replaced by polysporic plasmodia orientated longitudinally within affected fibres. In most cases, the muscle fibres were infected by single plasmodia, although occasionally multiple infections with numerous plasmodia developing inside the muscle fibres were noticed. Surrounding the parasitic plasmodia, a 'pseudocyst' or a thin layer of host connectives tissue and adjacent muscle fibres was

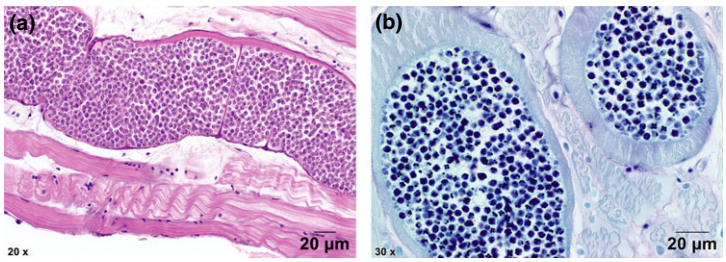

Figure 1 Skeletal muscle in lumpfish. Light microscopy. (a) The white muscle fibres were infected by polysporic plasmodia of *Kudoa islandica*. Note little host response associated with the parasitic infection. (b) Giemsa staining revealed that spores within the plasmodia were stellate in shape and contained four pyriform polar capsules.

observed. There was little host response associated with the parasitic infection (Fig. 1a). Giemsa staining revealed that spores within the plasmodia were stellate in shape and contained four pyriform polar capsules (Fig. 1b).

Gills. All fish showed multifocal and moderate to severe chronic gill inflammation, with fusion of lamella and hyperplasia of mucous cells. Several parasites were seen associated with the lesions: metacercaria of *Cryptocotyle* sp. (6/10) (Fig. 2b,c), protozoan ciliates (suspected *Trichodina* sp.) (9/10) (Fig. 2a), *Gyrodactylus* sp. (2/10) (Fig. 2d) and an unidentifiable suspected bacterial cyst in the gill lamella (5/10) (Fig. 2e).

Heart. Three fish had an extensive degree of necrosis and haemorrhages in the myocardium along the caudal face of the cardiac ventricle (Fig. 3a). In the affected areas, the nuclei of the cardiomyocytes were pyknotic and few inflammatory cells were present. One of the three affected hearts stained positive with von Kossa, indicating calcification (Fig. 3b).

Lesions in other tissues (spleen, liver, kidney and pancreas). Nine of ten fish showed extensive necrosis in the liver and spleen and multifocal kidney necrosis (Fig. 3c). Oedema was observed in glomeruli and kidney interstitium (Fig. 3d). Several fish had mild calcium deposits within the kidney tubules. In one fish, intracellular Gram-negative parasites were detected in the spleen (Fig. 3e) and the kidney. No lesions were observed in other organs (pancreas and intestine).

Confocal laser scanning microscopy

Structures assumed to be microsporidian spores were detected in all the examined tissues, except for skin and muscle. The brightly fluorescing, ovally shaped spores appeared to be located intracellularly and were mainly seen in pairs or as clusters, ranging from three to eight spores per cluster (Fig. 4a,b). The spores were most abundant in spleen and kidney, while more sparingly present in heart, liver, intestine and gills. In the latter four organs, the spores mainly seemed to be associated with blood or blood vessels.

Molecular analyses

No virus was detected in any of the samples. For the PCR targeting *Nucleospora cyclopteri*, five positive samples (three from gills and two from hearts) from the first PCR were analysed in the second nested PCR. All these five analyses resulted in amplification of one single band of the targeted size. Both these and all positive samples from the first round were sequenced. The assembled and edited sequences were 822 bp in total. The BLASTN search gave a 100% sequence identity to *N. cyclopteri* (Accession Number KC203457) except that some sequences contained one and the same ambiguous signal (A/G) in position 1365 related to KC203457. This was not followed up further in this study.

The PCR targeting *Kudoa islandica* gave multiple bands and only three PCR products from gill samples were considered good enough for sequencing. The assembled and edited sequences were 646 bp and were all identical. The BLASTN search gave a 100% sequence identity to *K. islandica* (KJ451388).

The PCR targeting *Gyrodactylus* spp. was positive for three gill samples and all three samples were sequenced. The assembled and edited sequences were 548 bp in total and contained three ambiguous bases. The BLASTN search gave no identical hits, but the closest hit was *G. flesi*

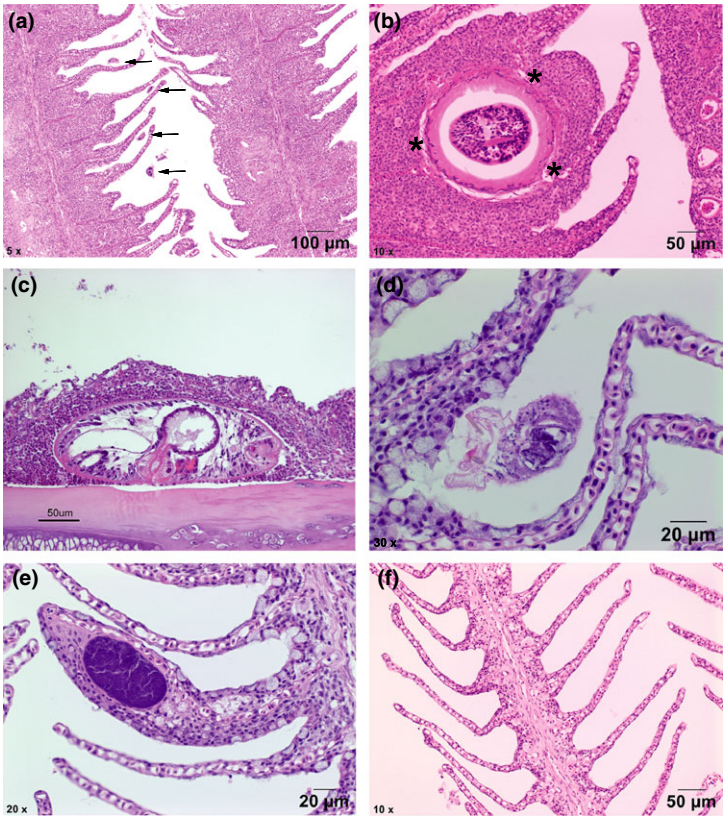

Figure 2 Gill inflammation in lumpfish. Light microscopy. (a) Severe chronic gill inflammation, with fusion of lamella and hyperplasia of mucous cells. Note presence of protozoan ciliates (suspected *Trichodina* sp) between lamella (arrows). (b) Encysted metacercaria of *Cryptocotyle* sp. Note severe inflammatory reaction around the cyst (*). (c) Longitudinal section of *Cryptocotyle* sp. (d) *Gyrodactylus* sp. between lamella. (e) Unidentifiable cyst in the gill lamella. (f) Normal lumpfish gill.

(AY338454) and *G. robustus* (AY278040) (94% identity). However, based on host identity and sequence comparison with other specimens from *C. lumpus* morphologically identified as *G. cyclopteri* (unpublished results), it is assumed that the species might be *G. cyclopteri* Scyborskaya, 1948 and the sequence is submitted to GenBank (KP090176) under this name.

Discussion

We here describe a case of a multispecies parasitic infection in farmed lumpfish in northern Norway. Several infectious agents were detected: A newly described myxozoan parasite, *Kudoa islandica* (Kristmundsson & Freeman 2014), was confirmed by histology and PCR as the cause of the muscle lesions. In addition, a newly described microsporidian *Nucleopora cyclopteri* (Freeman *et al.* 2013) was detected in gills and hearts using PCR, and microsporidian spores were seen in histological sections of gills and internal organs using Calcofluor White. Several other branchial parasites (*Cryptocotyle* sp., protozoon ciliates and *Gyrodactylus cyclopteri*) were also diagnosed. To the best of our knowledge, this is the first description of *K. islandica* and *N. cyclopteri* infecting farmed lumpfish in Norway.

Mortality started in summer, 18 months after sea transfer and a few non-specific signs like erratic swimming behaviour and loss of weight were observed. At histology, obvious and consistent lesions were recorded in muscle, gills and internal organs, and several parasites were seen associated with the lesions. In muscle, the histopathological findings were similar to those previously described from wild fish infected with *K. islandica* (Kristmundsson & Freeman 2014). The parasites were located intracellularly in white muscle fibres and multiple plasmodia were detected inside each single myofibre. No host response was detected in relation to the presence of the parasite. However, the degree of severity observed in this case was considerably milder than the infection observed in wild Icelandic lumpfish and could explain why no macroscopical muscle lesions or myoliquefaction was described. Some kudoids have been found infecting other organs (Moran *et al.* 1999;

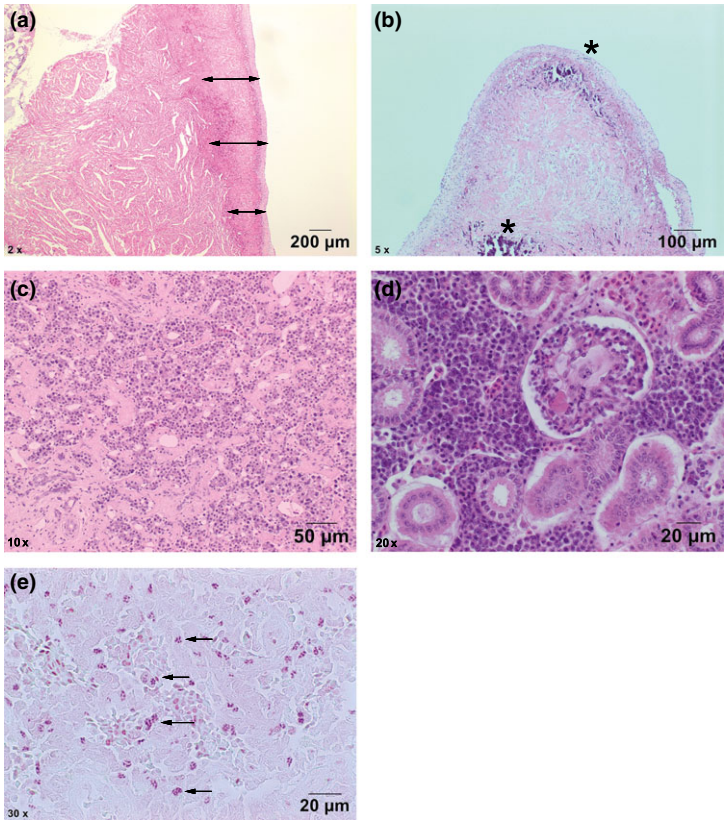

Figure 3 Internal organs in lumpfish. Light microscopy. (a, b) Hearts. Extensive necrosis and haemorrhages in the myocardium (arrow) along the caudal face of the cardiac ventricle. One heart had calcification in the necrotic areas (*). (c) Severe necrosis in the liver. (d) Kidney. Oedema was observed in glomeruli and kidney interstitium. (e) Spleen. Intracellular parasites were detected using Gram staining (arrows).

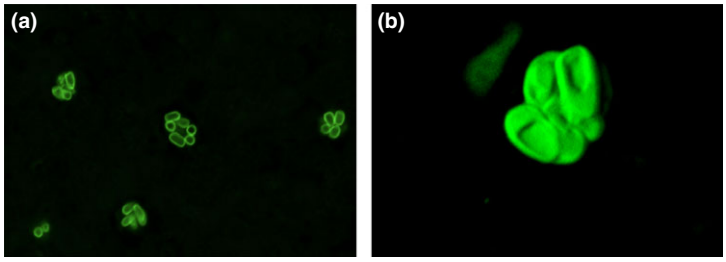

Figure 4 Microsporidian-infected cells stained with Calcofluor white. (a) Spleen. The brightly fluorescing, ovally shaped spores were located intracellularly and were mainly seen in pairs or as clusters, ranging from three to eight spores per cluster. The spores were most abundant in spleen and kidney. (b) Higher magnification of microsporidian spores.

Blaylock, Bullard & Whipps 2004). In the present case, although lesions were described in internal organs and *K. islandica* was detected in three of ten analysed hearts using PCR, we were not able to see *Kudoa* spores systemically using different special stains (Gram, PAS and Giemsa). *Kudoa* is of concern to both aquaculture and commercial fisheries because of the financial losses due to post-mortem myoliquefaction or 'soft flesh', but in general *Kudoa* infections do not appear to compromise the fish health (Moran *et al.* 1999). Based on the results in our case study, especially the low degree of infection and the lack of host response towards the myxosporidian, we do not consider *K. islandica,* the primary cause of the high mortality.

Nucleospora cyclopteri was detected in gills, but also systemically in hearts using PCR, in 5 of 10 sampled fish. Massive numbers of microsporidian spores were seen in kidney and spleen in all Calcofluor selected slides. Macroscopically, white nodules were described in the kidney and this is in accordance with previous descriptions of this microsporidian in lumpfish in Iceland and Canada

(Mullins *et al.* 1994; Freeman *et al.* 2013). In the present case, the histopathological lesions, most commonly found, were extensive areas of necrosis and oedema in the kidney interstitial tissue and nephrons. In addition, severe necrosis and degeneration were observed in liver and spleen in the majority of the fish sampled. In three of ten analysed hearts, focal and extensive degree of necrosis and haemorrhages was observed in the caudal part of the cardiac ventricle. While necrosis and lymphocyte infiltration in the kidney were the main lesions in the descriptions from wild lumpfish in Iceland, and captive lumpfish from Canada, cardiac lesions had not been reported previously associated with *N. cyclopteri* infection.

Confocal laser scanning microscopy after staining with Calcofluor White M2R was able to detect microsporidian-like organisms at variable abundance in kidney, spleen, gills, heart, liver and intestine. As reported by Freeman *et al.* (2013), the spores mostly appeared to be located intracellularly, mostly in clusters but also single ones. The method served to display the morphology and distribution of the parasites in the various tissues. It may also be a more sensitive method than regular histology to show presence of microsporidian spores in samples where pathology is less evident. Moreover, the possibility of 3D imaging of the organism *in situ* may prove useful in studies of life cycle and pathogenesis.

In the present case, the diagnosis was challenging due to co-infection with different parasite species in the affected tissues. Parasitic diseases can be understood in many cases as multifactorial, where parasites and hosts are in a dynamic and instable equilibrium that can balance towards disease due to the influence of environmental factors or demanding and stressful host conditions (Cordero del Campillo *et al.* 1999). In this field study, different parasites were causing severe gill damage and it is reasonable to believe that this eventually led to respiratory distress and contributed to the observed mortality. High mortality in the present case was mainly attributed to the newly described microsporidian species *N. cyclopteri* (Freeman *et al.* 2013), due to massive amounts of spores in internal organs associated with the lesions. In humans, microsporidiosis has been described both in immunosuppressed and immunocompetent patients producing subclinical to lethal infections (Didier & Weiss 2011). Several authors have hypothesized that microsporidia can be present at low intensity in healthy individuals. In Atlantic salmon, it has been suggested that infection with *D. lepeoptherii* may be latent in immunocompetent fish, becoming acute during periods of infection with other pathogens or stress situations (Freeman & Sommerville 2011). The precise role of *N. cyclopteri* in causing disease has to be confirmed by challenge experiments and epidemiological studies. Further investigations needs to be carried out to estimate the prevalence of this systemic microsporidiosis, a potential threat under stress conditions or suboptimal welfare conditions.

In summary, we have described the pathological findings of a *K. islandica* and *N. cyclopteri* co-infection in farmed lumpfish, *Cyclopterus lumpus* L., in Norway and compared the findings with previous descriptions from Iceland and Canada. This study expands the knowledge on lumpfish pathology, a fish species in rapid expansion in the aquaculture industry, and contributes to a better understanding of diseases in this species.

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